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<b>(21) International Application Number:</b> PCT/US99/06098 <b>(22) International Filing Date:</b> 19 March 1999 (19.03.99) <b>(30) Priority Data:</b> 60/079,124                      23 March 1998 (23.03.98)                      US 60/081,569                      13 April 1998 (13.04.98)                      US <b>(71) Applicant:</b> GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). <b>(72) Inventors:</b> DE SAUVAGE, Frederic, J.; 187 Shooting Star Isle, Foster City, CA 94404 (US). KLEIN, Robert, D.; 1044 Webster Street, Palo Alto, CA 94301 (US). PHILLIPS, Heidi, S.; 15 Pine Avenue, San Carlos, CA 94070 (US). ROSENTHAL, Arnon; 40 Tulip Court, Burlingame, CA 94010 (US). <b>(74) Agents:</b> TORCHIA, Timothy, E. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> GFR $\alpha$ 3 AND ITS USES  <b>(57) Abstract</b>  <p>The present invention relates to nucleotide sequences, including expressed sequence tags (ESTs), oligonucleotide probes, polypeptides, vectors and host cells expressing, and immunoadhesions and antibodies to mammalian GFR<math>\alpha</math>3, a novel <math>\alpha</math>-subunit receptor of the GDNF (i.e. GFR) receptor family. It further relates to an assay for measuring activation of a <math>\alpha</math>-subunit receptor by detecting tyrosine kinase receptor activation (i.e., autophosphorylation) or other activities related to ligand-induced <math>\alpha</math>-subunit receptor homo-dimerization or homo-oligomerization.</p>		

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## GFR $\alpha$ 3 AND ITS USES

### TECHNICAL FIELD

The present invention relates generally to the identification and isolation of novel DNA and to the recombinant production of novel polypeptides which are characterized by the presence of GFR $\alpha$ 3 sequences, an  $\alpha$ -subunit receptor. It further relates to an assay for measuring ligand-induced activation of an  $\alpha$ -subunit receptor by detecting autophosphorylation of a kinase domain of an  $\alpha$ -receptor- receptor protein tyrosine kinase (rPTK) fusion using a kinase receptor activation, enzyme-linked immunosorbent assay (KIRA ELISA) or by other means to detect  $\alpha$ -subunit homodimerization.

### INTRODUCTION

#### BACKGROUND

Neurotrophic factors such as insulin-like growth factors, nerve growth factor, brain-derived neurotrophic factor, neurotrophin-3, -4/5 and -6, ciliary neurotrophic factor, GDNF, and neurturin have been proposed as potential means for enhancing specific neuronal cell survival, for example, as a treatment for neurodegenerative diseases such as amyotrophic lateral sclerosis, Alzheimer's disease, stroke, epilepsy, Huntington's disease, Parkinson's disease, and peripheral neuropathy. It would be desirable to provide additional therapy for this purpose. Protein neurotrophic factors, or neurotrophins, which influence growth and development of the vertebrate nervous system, are believed to play an important role in promoting the differentiation, survival, and function of diverse groups of neurons in the brain and periphery. Neurotrophic factors are believed to have important signaling functions in neural tissues, based in part upon the precedent established with nerve growth factor (NGF). NGF supports the survival of sympathetic, sensory, and basal forebrain neurons both *in vitro* and *in vivo*. Administration of exogenous NGF rescues neurons from cell death during development. Conversely, removal or sequestration of endogenous NGF by administration of anti-NGF antibodies promotes such cell death (Heumann, *J. Exp. Biol.*, 132:133-150 (1987); Hefti, *J. Neurosci.*, 6:2155-2162 (1986); Thoenen *et al.*, *Annu. Rev. Physiol.*, 60:284-335 (1980)).

Additional neurotrophic factors related to NGF have since been identified. These include brain-derived neurotrophic factor (BDNF) (Leibrock, *et al.*, *Nature*, 341:149-152 (1989)), neurotrophin-3 (NT-3) (Kaisho, *et al.*, *FEBS Lett.*, 266:187 (1990); Maisonpierre, *et al.*, *Science*, 247:1446 (1990); Rosenthal, *et al.*, *Neuron*, 4:767 (1990)), and neurotrophin 4/5 (NT-4/5) (Berkmeier, *et al.*, *Neuron*, 7:857-866 (1991)).

Neurotrophins, similar to other polypeptide growth factors, affect their target cells through interactions with cell surface receptors. According to current understanding, two kinds of transmembrane glycoproteins act as receptors for the known neurotrophins. Equilibrium binding studies have shown that neurotrophin-responsive neuronal cells possess a common low molecular weight (65,000 - 80,000 Daltons), a low affinity receptor typically referred to as p75<sup>LNGFR</sup> or p75, and a high molecular weight (130,000-150,000 Dalton) receptor. The high affinity receptors are members of the trk family of receptor tyrosine kinases.

Receptor tyrosine kinases are known to serve as receptors for a variety of protein factors that promote cellular proliferation, differentiation, and survival. In addition to the trk receptors, examples of other receptor tyrosine kinases include the receptors for epidermal growth factor (EGF), fibroblast growth factor (FGF), and

platelet-derived growth factor (PDGF). Typically, these receptors span the cell membrane, with one portion of the receptor being intracellular and in contact with the cytoplasm, and another portion of the receptor being extracellular. Binding of a ligand to the extracellular portion of the receptor induces tyrosine kinase activity in the intracellular portion of the receptor, with ensuing phosphorylation of various intracellular proteins involved in cellular signaling pathways.

Glial cell line-derived neurotrophic factor ("GDNF") and Neurturin ("NTN") are two, recently identified, structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin *et al. Science* 260:1130-1132 (1993); Henderson *et al. Science* 266:1062-1064 (1994); Buj-Bello *et al., Neuron* 15:821-828 (1995); Kotzbauer *et al. Nature* 384:467-470 (1996)). Recently, GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked protein (designated GDNFR $\alpha$ ; also designated GFR- $\alpha$ -1) and the transmembrane receptor tyrosine kinase Ret (Treanor *et al. Nature* 382:80-83 (1996); Jing *et al. Cell* 85:1113-1124 (1996); Trupp *et al. Nature* 381:785-789 (1996); Durbec *et al. Nature* 381:789-793 (1996)). The NTN signal is transmitted via GFR $\alpha$ 2, which is also Ret associated.

Membrane-bound proteins and receptors can play an important role in the formation, differentiation and maintenance of multicellular organisms. The fate of many individual cells, e.g., proliferation, migration, differentiation, or interaction with other cells, is typically governed by information received from other cells and/or the immediate environment. This information is often transmitted by secreted polypeptides (for instance, mitogenic factors, survival factors, cytotoxic factors, differentiation factors, neuropeptides, and hormones) which are, in turn, received and interpreted by diverse cell receptors or membrane-bound proteins. Such membrane-bound proteins and cell receptors include, but are not limited to, cytokine receptors, receptor kinases, receptor phosphatases, receptors involved in cell-cell interactions, and cellular adhesion molecules like selectins and integrins. For instance, transduction of signals that regulate cell growth and differentiation is regulated in part by phosphorylation of various cellular proteins. Protein tyrosine kinases, enzymes that catalyze that process, can also act as growth factor receptors. Examples include fibroblast growth factor receptor and nerve growth factor receptor.

Membrane-bound proteins and receptor molecules have various industrial applications, including as pharmaceutical and diagnostic agents. Receptor immunoadhesins, for instance, can be employed as therapeutic agents to block receptor-ligand interaction. The membrane-bound proteins can also be employed for screening of potential peptide or small molecule inhibitors of the relevant receptor/ligand interaction.

The aberrant expression or uncontrolled regulation of any one of these receptor tyrosine kinases can result in different malignancies and pathological disorders. Therefore, there exists a need to identify means to regulate, control and manipulate receptor tyrosine kinases ("RTK"), their ligands, or their  $\alpha$ -subunit receptor molecules, e.g., GPI-linked  $\alpha$ -subunit receptors, to which they associate, in order to provide new and additional means for the diagnosis and therapy of receptor tyrosine kinase pathway-related disorders and cellular processes. The present application provides the clinician and researcher with such means by providing new molecules that are specific for interacting with certain receptor genes and their gene products. These compounds and their methods of use, as provided herein, allow exquisite therapeutic control and specificity.

Accordingly, it is one object of the present invention to provide an improved therapy for the prevention and/or treatment of neurological conditions and other conditions in which certain neurotrophic signaling pathways play a role.

#### SUMMARY

5 Applicants have identified a family of cDNAs that encode a novel human polypeptide or its homologs, designated in the present application as "GFR $\alpha$ 3 ." The GFR $\alpha$ 3 is an  $\alpha$ --subunit receptor, a receptor that complexes with a beta subunit receptor in response to ligand binding. A--subunits provide the ligand binding component and the beta subunit provides the catalytic signal transduction activity, such as tyrosine kinase activity. GFR $\alpha$  receptor family members complex with a beta subunit receptor referred to as  
10 Ret. This hetero-complex results in signal transduction. The present invention is based in part on the novel finding that the  $\alpha$ -subunit can dimerize upon binding ligand, and further the dimerization can activate a kinase activity of a kinase catalytic domain fused to the ligand-binding domain of the  $\alpha$ --subunit receptor.

In one embodiment, the invention provides an isolated nucleic acid molecule having at least about 65% sequence identity to (a) a nucleic acid sequence encoding a GFR $\alpha$ 3 polypeptide comprising the sequence  
15 of amino acids 27 to 400 of SEQ ID NO: 15, amino acids 27 to 369 of SEQ ID NO: 17 or amino acids 27 to 374 of SEQ ID NO: 5 or (b) the complement of the nucleic acid molecules of (a). In another embodiment, the nucleic molecule sequence above comprises a ligand-binding domain of a GFR $\alpha$ 3 polypeptide of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20, or their complementary nucleic acids. The isolated nucleic acid comprises a  
20 GFR $\alpha$ 3 encoding sequence which preferably hybridizes under stringent conditions to nucleic acid sequences encoding a GFR $\alpha$ 3 polypeptide of the invention. The sequence identity preferably is at least about 75%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%. In one aspect, the encoded polypeptide has at least about 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least about 95% sequence identity with a polypeptide having  
25 amino acid residues 27 to 400 of SEQ ID NO: 15, amino acids 27 to 369 of SEQ ID NO: 17, amino acids 27 to 374 of SEQ ID NO: 5, a ligand-binding domain of a GFR $\alpha$ 3 polypeptide of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20. Preferably the identity is to amino acid residues 27 to 400 of SEQ ID NO: 15 and DNA encoding it. In a further embodiment, the isolated nucleic acid molecule comprises DNA encoding a GFR $\alpha$ 3 polypeptide  
30 having amino acid residues 27 to 400 of SEQ ID NO: 15, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. In another aspect, the invention provides a nucleic acid of the full length protein of clone DNA48613, DNA48614 or murine GFR $\alpha$ 3 (clone 13), deposited with the ATCC under accession numbers ATCC 209752 (Designation: DNA48613-1268), ATCC 209751 (Designation: DNA48614-1268), and ATCC  
35 \_\_\_\_\_, respectively, on April 07, 1998.

In yet another embodiment, the invention provides a vector comprising DNA encoding GFR $\alpha$ 3 polypeptide. A host cell comprising such a vector is also provided. By way of example, the host cells may be CHO cells, *E. coli*, or yeast. A process for producing GFR $\alpha$ 3 polypeptides is further provided and

comprises culturing host cells under conditions suitable for expression of GFR $\alpha$ 3 and recovering the same from the cell culture.

In yet another embodiment, the invention provides isolated GFR $\alpha$ 3 polypeptide. In particular, the invention provides isolated native sequence GFR $\alpha$ 3 polypeptide, which in one embodiment, includes an amino acid sequence comprising residues 27 to 400 SEQ ID NO:15. Native GFR $\alpha$ 3 polypeptides with or without the native signal sequence (amino acids 1 to 26) in SEQ ID NO: 15, and with or without the initiating methionine are specifically included. In yet another embodiment is provide a polypeptide comprising a sequence of amino acid residues 27 to 400 of SEQ ID NO: 15, amino acids 27 to 369 of SEQ ID NO: 17, amino acids 27 to 374 of SEQ ID NO: 5, a ligand-binding domain of a GFR $\alpha$ 3 polypeptide of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20. Alternatively, the invention provides a GFR $\alpha$ 3 polypeptide encoded by the nucleic acid deposited under the above accession numbers. The polypeptide optionally is lacking the hydrophobic sequence associated with GPI-anchoring.

In yet another embodiment, the invention provides chimeric molecules comprising a GFR $\alpha$ 3 polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises a GFR $\alpha$ 3 polypeptide fused to an epitope tag sequence or an Fc region of an immunoglobulin. The chimeric molecules can comprise the ligand-binding domain of an  $\alpha$ -subunit receptor, the intracellular catalytic domain of a tyrosine kinase receptor, and a flag epitope.

In yet another embodiment, the invention provides an antibody which specifically binds to GFR $\alpha$ 3 polypeptide. Optionally, the antibody is a monoclonal antibody.

In view of the surprising finding herein that the  $\alpha$ --subunit receptor can dimerize upon ligand binding, and further that such dimerization can activate a kinase domain fused the  $\alpha$ - subunit receptor, a method is provided herein to measure ligand-induced  $\alpha$ - subunit receptor activation, i.e. homo-dimerization or homo-oligomerization. In one embodiment is provided a sensitive, reliable assay that measures agonist- or ligand-induced  $\alpha$ --subunit receptor activation, i.e., homo-dimerization or homo-oligomerization, preferably by measuring receptor protein tyrosine kinase (rPTK) autophosphorylation of a polypeptide fusion comprising the ligand binding domain of an  $\alpha$ --subunit receptor and the intracellular catalytic domain of a receptor protein tyrosine kinase. The construct can further optionally comprise a flag epitope to facilitate trapping and detection of the activated (e.g., dimerized, phosphorylated)  $\alpha$ --subunit receptor. The assay is desirably useful for qualitatively and quantitatively measuring  $\alpha$ --subunit receptor activation as well as facilitating identification and characterization of potential agonists and antagonists for a selected  $\alpha$ --subunit receptor. It is a further object of the invention to provide an assay which enables ligand-receptor interactions to be studied for any selected  $\alpha$ - subunit receptor, and preferably a GFR $\alpha$  subunit receptor.

This assay must have a capacity for high throughput, that is, the ability to reliably evaluate large numbers of samples in a relatively short period of time (e.g., in one day). The assay ideally does not use radioactive materials and is also amenable to automation.

In at least one embodiment of the invention is provided a generic assay which enables a  $\alpha$ --subunit receptor of interest to be studied, regardless of whether or not a receptor-specific capture agent having the

desired characteristics is available. Furthermore, it is an object of the invention to provide an assay which substantially represents the ligand-binding activity of the  $\alpha$ - subunit receptor *in situ*. This is desirable insofar as it reduces the possibility that altered interactions between the receptor and the ligand may occur as a consequence of the receptor not being membrane-bound. In one embodiment of the assay is provided a method for measuring ligand binding by detecting serine-threonine kinase phosphorylation, phosphorylation of intracellular kinases and phosphatase activity of a catalytic domain fused to the  $\alpha$ - subunit receptor. Accordingly, the invention provides an assay for measuring activation or ligand binding of an  $\alpha$ - subunit receptor construct chimera by detecting its homo-dimerization or homo-oligomerization by in turn measuring kinase or phosphatase activity (*i.e.*, by autophosphorylation) of catalytic domain that is fused to the ligand-binding domain of an  $\alpha$ --subunit receptor of interest.

The assay can be divided into two major stages, each of which is generally performed in separate assay plates. The first stage of the assay involves activating the  $\alpha$ - subunit receptor construct, preferably in a KIRA stage of the assay. The second stage of the assay involves measuring receptor construct activation. Conveniently, this is achieved using an enzyme-linked immunosorbent assay (ELISA) to measure receptor construct activation.

The KIRA stage of the assay involves activating a  $\alpha$ --subunit receptor- kinase receptor fusion construct which is located in the cell membrane of an eukaryotic cell such that the extracellular domain of the  $\alpha$ --subunit receptor faces the external milieu of the cell, a transmembrane domain is located in the cell membrane and the catalytic kinase domain is located intracellularly. This stage of the overall assay involves steps (a) to (c) below:

(a) The first solid phase (*e.g.*, a well of a first assay plate) is coated with a substantially homogeneous population of cells (usually a mammalian cell line) so that the cells adhere to the solid phase. Often, the cells are adherent and thereby adhere naturally to the first solid phase. In one embodiment of the invention, the cells have been transformed with DNA encoding a polypeptide receptor construct comprising an  $\alpha$ --subunit receptor ligand-binding domain fused to a catalytic kinase domain, or a "receptor construct" defined further below, which DNA is expressed by the cells such that the receptor or receptor construct is suitably positioned in the cell membranes thereof.

The receptor construct further, and preferably, comprises a fusion with a flag polypeptide. The flag polypeptide is recognized by the capture agent, often a capture antibody, in the ELISA part of the assay. Use of a receptor construct as disclosed herein is particularly advantageous since it provides a "generic" assay wherein autophosphorylation of any kinase receptor domain can be measured, regardless of whether or not a receptor-specific capture agent having the required characteristics is available. Often, the receptor construct is a fusion protein comprising the ECD of a selected  $\alpha$ --subunit receptor, the catalytic ICD (and possibly the transmembrane domain) of another well characterized tyrosine kinase (*e.g.*, the Rse receptor).

(b) An analyte is then added to the wells having the adhering cells, such that the receptor construct is exposed to (or contacted with) the analyte. This assay enables identification of agonist and antagonist ligands for the  $\alpha$ --subunit receptor of interest. In order to detect the presence of an antagonist ligand which blocks binding and/or activation of the receptor by an agonist ligand, the adhering cells are exposed to the

suspected antagonist ligand first and then to the agonist ligand (or to a mixture of the agonist and antagonist) so that competitive inhibition of receptor binding and activation can be measured. Also, the assay can identify an antagonist which binds to the agonist ligand and thereby reduces or eliminates its ability to bind to, and activate, the kinase domain. To detect such an antagonist, the suspected antagonist and the agonist for the receptor are incubated together and the adhering cells are then exposed to this mixture of ligands.

(c) Following exposure to the analyte, the adhering cells are solubilized using a lysis buffer (which has a solubilizing detergent therein) and gentle agitation, thereby releasing cell lysate which can be subjected to the ELISA part of the assay directly, without the need for concentration or clarification of the cell lysate. Thus, this assay provides a significant improvement over assays described by Knutson and Buck, *supra*, Klein *et al.*, *supra*, and Hagino *et al.*, *supra*, insofar as it is surprisingly unnecessary to concentrate the cell lysate prior to the ELISA. Furthermore, unlike the other assays, in the instant assay the cells can be lysed in lysis buffer using gentle agitation without the need for homogenizing, centrifuging or clarifying the cells. The cell lysate thus prepared is then ready to be subjected to the ELISA stage of the assay. It has been discovered that, surprisingly, the first assay plate can be stored at freezing temperatures (*i.e.*, at about -20° to -70°C) for significant periods of time (at least 6 months) before the ELISA stage of the assay. This is a significant finding insofar as the KIRA and ELISA stages of the assay can be performed on separate days.

The ELISA component of the assay comprises steps (d) to (h), described below.

(d) As a first step, the second solid phase (usually a well of an ELISA microtiter plate) is coated with a capture agent (often a capture antibody) which binds specifically to the receptor construct, preferably to an optionally present flag polypeptide. Coating of the second solid phase is carried out so that the capture agent adheres to the second solid phase. The capture agent is generally a monoclonal antibody, but, as is described in the examples herein, polyclonal antibodies may also be used.

(e) The cell lysate obtained in step (c) of the above-mentioned KIRA stage of the assay is exposed to, or contacted with, the adhering capture agent so that the receptor construct adheres to (or is captured in) the second solid phase. Unlike the assay of Klein *et al.*, the instant assay does not require the ligand for the receptor as well as kinase inhibitors to be present to achieve suitable immobilization of the receptor or receptor construct to the second solid phase.

(f) A washing step is then carried out, so as to remove unbound cell lysate, leaving the captured receptor or receptor construct.

(g) The adhering or captured receptor construct is then exposed to, or contacted with, an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor domain. In the preferred embodiment, the anti-phosphotyrosine antibody is conjugated (directly or indirectly) to an enzyme which catalyses a color change of a non-radioactive color reagent. Accordingly, phosphorylation of the receptor can be measured by a subsequent color change of the reagent. The enzyme can be bound to the anti-phosphotyrosine antibody directly, or a conjugating molecule (*e.g.*, biotin) can be conjugated to the anti-phosphotyrosine antibody and the enzyme can be subsequently bound to the anti-phosphotyrosine antibody via the conjugating molecule.



(h) Finally, binding of the anti-phosphotyrosine antibody to the captured receptor construct is measured, *e.g.*, by a color change in the color reagent.

The invention also pertains to a Rse.flag reagent which is particularly useful for use in the KIRA ELISA assay. The Rse.flag reagent is a polypeptide comprising a fusion of a flag polypeptide (usually the gD flag described herein) to the carboxyl terminus of the intracellular domain of the Rse rPTK. Generally, the transmembrane domain of Rse and the extracellular domain of another rPTK of interest are also present in the fusion polypeptide reagent. The nucleic acid encoding this reagent and a cell transformed therewith are also claimed.

In yet a further aspect, the invention relates to a kit which can be used in the KIRA ELISA disclosed above which comprises an anti-flag polypeptide capture agent (*e.g.* a capture antibody) which is usually bound to the second solid phase as described herein, and a receptor construct. Thus, the kit generally provides an ELISA microtiter plate having an anti-flag polypeptide capture antibody adhering to a well thereof. Optionally, the kit also provides an anti-phosphotyrosine antibody which is often labelled, or reagents for labelling the anti-phosphotyrosine antibody are supplied with the kit. Sometimes, a homogeneous population of cells which have been transformed with a receptor construct as described herein are also provided with the kit. The kit can also suitably includes instructions for carrying out the KIRA ELISA.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-B shows the nucleotide sequence and deduced amino acid sequence of a native sequence of murine GFR $\alpha$ 3.

Figure 2 shows the alignment of the amino acid sequences for murine GFR $\alpha$ 3 (SEQ ID NO: 5), rat GFR $\alpha$ 1 (SEQ ID NO: 8) and rat GFR $\alpha$ 2 (SEQ ID NO: 9). The N-terminal signal peptides are indicated. The C-terminal hydrophobic sequences associated with GPI-anchoring are overlined. Asterisks indicate the amino acids for GPI-anchor attachment. Potential glycosylation sites are marked by shaded boxes. Conserved identical residues are boxed.

Figure 3 shows the alignment comparison between murine and human GFR $\alpha$ 3 amino acid sequences. Conserved residues are boxed.

Figure 4 shows the alignment comparison between human GFR $\alpha$ 3 (from DNA48613) and its splice variant (from DNA48614). Conserved sequences are boxed. The 30 amino acid deletion sequence is indicated.

Figures 5A-B shows the nucleic acid sequence alignment of the DNA sequence (SEQ ID NO: 14) encoding human GFR $\alpha$ 3 with DNAs (SEQ ID NO: 21 and SEQ ID NO: 22) encoding human GFR $\alpha$ 1 (SEQ ID NO: 6) and human GFR $\alpha$ 2 (SEQ ID NO: 7), respectively.

Figure 6 shows the amino acid sequence alignment of human GFR $\alpha$ 3 (SEQ ID NO: 15), human GFR $\alpha$ 1 (SEQ ID NO: 6) and human GFR $\alpha$ 2 (SEQ ID NO: 7).

Figure 7 presents multiple tissue Northern blots using GFR $\alpha$ 3 as a probe.

Figure 8 compares RNA expression localization determined by *in situ* hybridization using DNA probes specific for GFR $\alpha$ 1, GFR $\alpha$ 2 and GFR $\alpha$ 3.

Figures 9A-C present the results of ligand binding (rat GDNF, human neurturin (NTN) or Human persephin (PSN)) to IgG-tagged receptors GFR $\alpha$ 1 (Figure 9A), GFR $\alpha$ 2 (Figure 9B) or GFR $\alpha$ 3 (Figure 9C).

Figure 10 presents the proliferation of cells expressing recombinant chimeric GFR $\alpha$ 2-*mpl* in response to NTN or GDNF.

Figure 11 presents the auto-phosphorylation of recombinantly expressed receptor GFR $\alpha$ 2-Rse in response to NTN.

Figure 12 presents the assay for stimulation of receptors GFR $\alpha$ 2 or GFR $\alpha$ 3 by GDNF, NTN or PSN.

Figure 13 depicts the agonist activity of various anti-gD antibodies in a gD-GFR $\alpha$ -2-Rse KIRA assay.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

##### 10 I. Definitions

The terms "GFR $\alpha$ 3" "GFR $\alpha$ 3 polypeptide" and "GFR $\alpha$ 3-homolog" when used herein encompass native sequence GFR $\alpha$ 3 and GFR $\alpha$ 3 variants (which are further defined herein). The GFR $\alpha$ 3 may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods. A "native sequence GFR $\alpha$ 3" comprises a polypeptide having the same amino acid  
 15 sequence as a GFR $\alpha$ 3 derived from nature. Such native sequence GFR $\alpha$ 3 can be isolated from nature or can be produced by recombinant or synthetic means. The term "native sequence GFR $\alpha$ 3" specifically encompasses naturally-occurring truncated or secreted forms of GFR $\alpha$ 3 (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of GFR $\alpha$ 3. In one embodiment of the invention, the native sequence GFR $\alpha$ 3 is a mature or full-  
 20 length native sequence GFR $\alpha$ 3 comprising amino acids 1 to 400 of SEQ ID NO:15, with or without the N-terminal signal sequence, and with or without the initiating methionine at position 1.

"GFR $\alpha$ 3 variant" means an active GFR $\alpha$ 3 as defined below having at least about 75% amino acid sequence identity to (a) a DNA molecule encoding a GFR $\alpha$ 3 polypeptide, with or without its native signal sequence, or (b) the complement of the DNA molecule of (a). In a particular embodiment, the GFR $\alpha$ 3 variant  
 25 has at least about 80% amino acid sequence homology with the GFR $\alpha$ 3 having the deduced amino acid sequence shown in SEQ ID NO: 15 for a full-length native sequence GFR $\alpha$ 3. Such GFR $\alpha$ 3 variants include, for instance, GFR $\alpha$ 3 polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of SEQ ID NO:15. Preferably, the nucleic acid or amino acid sequence identity is at least about 75%, more preferably at least about 80%, and even more preferably at least about 90%, and  
 30 yet even more preferably at least about 95%.

"Percent (%) amino acid sequence identity" with respect to the GFR $\alpha$ 3 sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the GFR $\alpha$ 3 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part  
 35 of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art

can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Percent (%) nucleic acid sequence identity" with respect to the GFR $\alpha$ 3 sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the GFR $\alpha$ 3 sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the GFR $\alpha$ 3 natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" DNA48613 nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the DNA48613 nucleic acid. An isolated DNA48613 nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated DNA48613 nucleic acid molecules therefore are distinguished from the DNA48613 nucleic acid molecule as it exists in natural cells. However, an isolated DNA48613 nucleic acid molecule includes DNA48613 nucleic acid molecules contained in cells that ordinarily express DNA48613 where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of

a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel et al., *Current Protocols in Molecular Biology*, Wiley Interscience Publishers, (1995).

"Stringent conditions" or "high stringency conditions", as defined herein, may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50EC; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42EC; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 42EC, with washes at 42EC in 0.2 x SSC (sodium chloride/sodium citrate) and 0.1% SDS; or (4) employ a buffer of 10% dextran sulfate, 2 x SSC and 50% formamide at 55EC, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55EC.

"Moderately stringent conditions" may be identified as described by Sambrook et al., *Molecular Cloning: A Laboratory Manual*, New York: Cold Spring Harbor Press, 1989, and include the use of washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent than those described above. An example of moderately stringent conditions is overnight incubation at 37EC in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50EC. The skilled artisan will recognize how to adjust the temperature, ionic strength, etc. as necessary to accommodate factors such as probe length and the like.

"rPTK" means a receptor protein tyrosine kinase.

"ECD", "TM domain" and "ICD" refer to the extracellular domain, transmembrane domain and intracellular domain of a rPTK, respectively.

"Kinase Receptor Activation" or "KIRA" when used throughout this application refers to the first stage of the instantly claimed assay wherein a cell-bound receptor construct (typically with a rPTK ICD domain) is exposed to a potential agonist/antagonist ligand which may (or may not) induce phosphorylation

of tyrosine residues in the intracellular domain of the rPTK portion of the receptor construct. The KIRA is generally carried out in the "first assay plate" as defined herein. U.S. Patent 5,766,863, and its corresponding WO publication, entitled "Kinase receptor activation assay" are hereby incorporated herein in their entirety for teaching a KIRA assay using a recombinantly expressed protein fusion of a receptor extracellular domain and a substitute enzymatic domain, e.g. tyrosine kinase domain.

"Enzyme-Linked Immunosorbent Assay" or "ELISA" refers to the second stage of the instantly claimed assay and involves measuring tyrosine phosphorylation of the kinase domain of the receptor construct. The ELISA is normally carried out in the "second assay plate" as disclosed in this application. The ELISA is a "sandwich ELISA" insofar as it involves capturing the receptor construct to the second solid phase (usually the well of an ELISA microtiter plate). ELISA assays generally involve the preparation of enzyme-antibody conjugates. The conjugated enzyme cleaves a substrate to generate a colored reaction product that can be detected spectrophotometrically. In this assay, the absorbance of the colored solution in individual microtiter wells is proportional to the amount of phosphotyrosines. A review of ELISA is found in Current Protocols in Molecular Biology, Vol. 2, chapter 11 (1991). While the term "ELISA" is used to describe the second stage of the instant assay, it is only a preferred embodiment of the invention, since, as disclosed herein, techniques other than enzymatic detection are available for measuring binding of the anti-phosphotyrosine antibody to the activated receptor.

The terms "tyrosine kinase", "tyrosine kinase receptor", "receptor protein tyrosine kinase" and "rPTK" are used interchangeably herein and refer to a protein having at least one phosphate accepting phenolic group in its ICD. The protein is usually a receptor insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and has one or more phosphate accepting tyrosine residues. Examples of tyrosine kinase receptors include the insulin receptor, insulin related receptor, epidermal growth factor receptor (EGF-R), platelet-derived growth factor receptors A and B (PDGF-R-A and PDGF-R-B), insulin-like growth factor 1 receptor (IGF-1-R), macrophage colony-stimulating factor receptor (M-CSF-R), HER2/*neu*/c-*erbB*-2 receptor, HER3/c-*erbB*-3 receptor, *Xmrk* receptor, IRR receptor, fibroblast growth factor (FGF) receptors *bek* and *flg*, c-*kit* receptor, Flk/kDR receptor, Rse receptor, the *Eph*, *Elk*, *Eck*, *Eek*, *Erk*, *Cek4*/*Mek4*/*HEK* and *Cek5* receptors, Axl receptor, hepatocyte growth factor receptor (HGF-R), Flt1 VEGF receptor, SAL-S1 receptor, HpTK 5 receptor, trkA receptor, trkB receptor, and trkC receptor. See, for example, Ullrich and Schlessinger Cell 81:203-212 (1990); Fantl *et al.*, Annu. Rev. Biochem. 62:453-481 (1993); Mark *et al.*, Journal of Biological Chemistry 269(14):10720-10728 (1994); and WO 93/15201.

The terms mentioned above encompass chimeric "receptor" molecules or "receptor constructs" or " $\alpha$ --subunit receptor constructs" which comprise at least the extracellular domain of a selected  $\alpha$ --subunit receptor, and the intracellular domain of a kinase receptor (preferably a rPTK), and optionally, the transmembrane domain of the same or another tyrosine kinase, and further optionally a flap epitope. Of course, the  $\alpha$ -receptor of interest can provide the transmembrane domain if it has one. The terms also encompass amino acid sequence variants and covalent derivatives of the various  $\alpha$ --subunit receptors and rPTKs kinase domains to which they are fused, provided they still display kinase phosphorylation activity in the KIRA ELISA. Therefore, the variants will generally have conservative amino acid alterations. The individual domains of the

$\alpha$ --subunit receptor kinase can be delineated based on sequence homology to known receptors in the relevant family and hydrophobicity plots. For example, the hydrophobic transmembrane domain can be readily determined and the ECD and ICD, when present, are usually amino-terminal and carboxyl terminal to the transmembrane domain or GPI-anchor, respectively. Conveniently, the transmembrane domain and ICD of the Rse receptor can be fused to the ECD of a  $\alpha$ --subunit receptor of interest, typically with the GPI-anchor sequence, thereby forming a chimeric receptor which is encompassed by the terms denoting a receptor construct as mentioned herein.

In the preferred embodiment, the  $\alpha$ --subunit receptor is selected from the group consisting of GFRa1, GFRa2, GFRa3, and GFRa4.

By "autophosphorylation" is meant activation of the catalytic kinase domain of the rPTK portion of the receptor construct, whereby at least one intrinsic tyrosine residue is phosphorylated. Generally, autophosphorylation will result when an agonist molecule binds to the extracellular domain of the  $\alpha$ --subunit receptor. Without being limited to any particular mechanism of action, it is thought that binding of the agonist molecule results in oligomerization of the receptor construct which causes activation of the catalytic kinase domain.

By "solid phase" is meant a non-aqueous matrix to which the cells (in the KIRA stage of the assay) or the capture agent (in the ELISA stage of the assay) can adhere. Usually, the solid phase comprises the well of an assay plate but the invention is by no means limited to this embodiment. For example, the solid phase can comprise a discontinuous solid phase of discrete particles. The particles can be porous and formed from a number of different materials, *e.g.*, polysaccharides (*e.g.* agarose), polyacrylamides, polystyrene, polyvinyl alcohol, silicones and glasses. For examples of suitable particulate solid phases, see U.S. Patent No. 4,275,149.

By "well" is meant a recess or holding space in which an aqueous sample can be placed. The well is provided in an "assay plate". The invention usually employs a "first assay plate" which is formed from a material (*e.g.* polystyrene) which optimizes adherence of cells (having the receptor or receptor construct) thereto. Generally, the individual wells of the first assay plate will have a high surface area to volume ratio and therefore a suitable shape is a flat bottom well (where the cells are adherent). The "second assay plate" is generally formed from a material (*e.g.* polystyrene) which optimizes adherence of the capture agent thereto. The second assay plate may have the same general construction and/or characteristics as the first assay plate. However, separate plates are used for the KIRA stage of the assay and the ELISA stage of the assay.

In the preferred embodiment of the invention, both the first assay plate and the second assay plate are "microtiter" plates. The term "microtiter" plate when used herein refers to an assay plate having between about 30 to 200 individual wells, usually 96 wells. Often, the individual wells of the microtiter plate will hold a maximum volume of about 250 $\mu$ l. Conveniently, the first assay plate is a 96 well polystyrene or plastic, cell culture microtiter plate (such as that sold by Becton Dickinson Labware, Lincoln Park, NJ), which allows for automation. Often, about 50 $\mu$ l to 300 $\mu$ l, more preferably 100 $\mu$ l to 200 $\mu$ l, of an aqueous sample comprising cell culture media with the cells suspended therein will be added to each well of the first assay plate in the KIRA stage of the assay. It is desirable to seed between about  $1 \times 10^4$  to  $3 \times 10^5$  cells per well. More

preferably,  $5 \times 10^4$  to  $1 \times 10^5$  cells per well are seeded. Usually, the second assay plate will comprise a polystyrene microtiter ELISA plate such as that sold by Nunc Maxisorp, Inter Med, Denmark.

The term "homogeneous population of cells" refers to a substantially homogeneous population of cells wherein at least about 80%, and preferably about 90%, of the cells in the population are of the same cell type.

5 Therefore, it is convenient to use a cell line. The cell line is a eukaryotic cell line, normally an animal cell line and desirably a mammalian cell line.

The cells have, or are transformed to produce, the selected receptor construct. Accordingly, the cell is transformed with a nucleic acid encoding the receptor construct and the nucleic acid is expressed so that the ECD of the receptor faces the external milieu of the cell, the transmembrane domain is located in the cell  
10 membrane and the kinase domain is located intracellularly. As a general proposition, a minimum number of about  $1 \times 10^4$  receptors/cell is required.

The term "adherent" when used herein to describe the cell, refers to a cell which naturally adheres to the first solid phase (often the well of the first assay plate), thereby forming a fairly uniform coating of the cells on the inside surface of the well. The uniform coating of cells generally forms following incubation of  
15 the cells in the wells of the first assay plate for about 8-16 hours. After incubation, non-adhering cells and cell culture medium are decanted off the first assay plate. Incubation is usually carried out at a temperature which is optimal for cell growth, *i.e.*, about 37°C. Examples of adherent cell lines include CHO cells (Urlaub and Chasin, Proc. Natl. Acad. Sci. USA 77:4216 (1980)), MCF-7 cells (ATCC HB 22), 293 cells (Graham *et al.*, J. Gen. Virol. 36:59 (1977)), Swiss albino 3T3 fibroblast cell line (ATCC No. CCL 92) and U937 macrophage  
20 cell line (ATCC No. CRL 1593).

A "flag polypeptide" comprises a short polypeptide which has enough residues to provide an epitope (preferably a linear epitope) against which a "capture agent" thereagainst can be made, yet is short enough such that it does not interfere with activity of the kinase domain or the ligand-binding domain. The flag polypeptide is also sufficiently unique so that the capture agent thereagainst does not bind to other reagents  
25 in the assay. Selection of a "unique" flag polypeptide sequence can be accomplished by comparing the sequence of a proposed flag polypeptide against other known sequences in Genbank or EMBL, for example. Suitable flag polypeptides generally have at least 6 amino acid residues and usually between about 8-80 amino acid residues (preferably between about 9-30 amino acid residues).

By "receptor construct" is meant a polypeptide which comprises a fusion of an  $\alpha$ -subunit receptor  
30 ligand-binding domain and a kinase receptor catalytic domain, and optionally a flag polypeptide as defined above. The flag polypeptide is provided at a location in the receptor construct such that: a) the flag polypeptide does not interfere with ligand binding to the receptor; b) the flag polypeptide does not interfere with autophosphorylation of the receptor and c) the flag polypeptide is presented in a suitable configuration so that it can bind to the capture agent in the ELISA stage of the assay. Often, the polypeptide flag will be  
35 present at the N-terminus of the receptor construct. Alternatively, the flag polypeptide may be present at the C-terminus of the receptor construct. An Rse.gD construct is preferred. The Rse construct disclosed herein is particularly useful, since the ICD (and optionally the transmembrane domain) thereof can be fused to the

ECD of a receptor of interest, thereby obviating the need to establish where the flag polypeptide should be located with respect to the receptor of interest.

"Rse.gD" refers to a receptor construct which has the Rse receptor protein tyrosine kinase ICD domain with the Herpes Simplex virus glycoprotein D (gD) flag polypeptide fused to the COOH-terminus thereof.

5 "Rse.flag reagent" refers to a polypeptide which comprises the ICD of the Rse receptor fused at its COOH-terminus to a flag polypeptide (normally the gD flag polypeptide). Sometimes, the TM domain of Rse with the ECD of an  $\alpha$ -subunit receptor of interest will also be present in the Rse.gD. reagent. "Receptor ECD/Rse.gD Chimera" refers to a fusion of the ECD of a  $\alpha$ -subunit receptor ligand-binding domain of interest to the TM and ICD domains of Rse which are fused COOH-terminally to the gD flag polypeptide.

10 By "capture agent" is meant a compound or agent which is able to adhere to the second solid phase, as herein defined, and which is selective for a receptor construct. Thus, the capture agent captures the receptor construct to the wells of the second assay plate. Usually, the capture agent binds selectively to the flag polypeptide which has been fused to the receptor of interest. Binding of the capture agent is not affected by the presence or absence of ligand bound to the receptor and does not induce receptor activation upon capture.

15 Furthermore, the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Means for selecting suitable capture agents are described herein. Generally, the capture agent will comprise an antibody (*e.g.*, an affinity purified polyclonal antibody or a monoclonal antibody), but other selective agents, such as streptavidin which binds selectively to the "strep-tag" polypeptide can also be used (see Schmidt *et al.*, Protein Engineering **6**(1):109-122 (1993)). Streptavidin can

20 be purchased commercially from Zymed Laboratories, S. San Francisco, CA, for example. Alternatively, the capture agent can comprise protein A (which binds specifically to immunoglobulins). In this embodiment of the invention, the activated receptor-construct present in the cell lysate is incubated with an antibody which binds specifically thereto, thereby forming a receptor-antibody complex. This complex can be captured by protein A by virtue of its specific binding to the antibody present in the complex. Protein A can be purchased

25 commercially from Pharmacia Biotech, Inc., Piscataway, New Jersey, for example.

In the most preferred embodiment, the capture agent is a monoclonal antibody which binds specifically to a flag polypeptide (which is present in the receptor construct). Examples of suitable flag polypeptides and their respective capture antibodies include the flu HA flag and its antibody 12CA5, (Field *et al.*, Mol. Cell. Biol. **8**:2159-2165 (1988)); the c-myc flag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan *et al.*, Molecular and Cellular Biology **5**(12):3610-3616 (1985)); as well as the Herpes Simplex virus glycoprotein D (gD) flag and the 5B6 antibody thereto (Paborsky *et al.*, Protein Engineering **3**(6):547-553 (1990) and Mark *et al.*, Journal of Biological Chemistry **269**(14):10720-10728 (1994)). Other flag polypeptides have been disclosed. Examples include the Flag-peptide (Hopp *et al.*, BioTechnology **6**:1204-1210 (1988)); the KT3 epitope peptide (Martin *et al.*, Science **255**:192-194 (1992)); an  $\alpha$ -tubulin epitope

30 peptide (Skinner *et al.*, J. Biol. Chem **266**:15163-15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth *et al.*, Proc. Natl. Acad. Sci. USA **87**:6393-6397 (1990)). Once the flag polypeptide has been selected as discussed above, a capture antibody thereto can be generated using the techniques disclosed herein.

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The term "analyte" refers to a compound or composition to be studied, usually to investigate its ability to activate (or prevent activation of) the  $\alpha$ --subunit receptor of interest. The analyte can comprise a bodily fluid (such as plasma or amniotic fluid) or a composition known to contain, or suspected of containing, a ligand for the tyrosine kinase receptor. The analyte can also comprise a cell which has a ligand to the  $\alpha$ --subunit receptor of interest.

"Ligand" when used herein refers to a molecule which is able to bind to the extracellular  $\alpha$ --subunit receptor of interest or to a known agonist thereof. The ligand will usually be an agonist or antagonist for the receptor.

By "agonist" is meant a molecule which is able activate the intracellular kinase domain of the receptor construct upon binding to the extracellular  $\alpha$ --subunit receptor portion. Often, the agonist will comprise a growth factor (*i.e.*, a polypeptide that is able to stimulate cell division). Exemplary growth factors include artemin, neurturin, GDNF and persephin. Alternatively, the agonist can be an antibody against the receptor or even its flag sequence as shown here in the Examples. However, other non-protein agonists such as small organic molecules are also encompassed by the invention.

By "antagonist" is meant a molecule which blocks agonist action. Usually, the antagonist will either: (a) bind to the  $\alpha$ --subunit receptor portion and thereby block binding and/or activation of the receptor by an agonist thereto (the antagonist may bind to the ECD of the receptor, but this is not necessarily the case) or (b) bind to the agonist and thus prevent activation of the receptor by the agonist. This assay facilitates the detection of both types of antagonist. The antagonist may, for example, comprise a peptide fragment comprising the receptor binding domain of the endogenous agonist ligand for the receptor. The antagonist may also be an antibody which is directed against the ECD of the receptor, or against a known agonist for the receptor. However, other non-protein molecules are also encompassed by this term.

The term "antibody" is used in the broadest sense, and can more specifically cover single anti-GFR $\alpha$ 3 monoclonal antibodies (including agonist, antagonist, and neutralizing antibodies) and anti-GFR $\alpha$ 3 antibody compositions with polyepitopic specificity. The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally-occurring mutations that may be present in minor amounts.

"Active" or "activity" for the purposes herein refers to form(s) of GFR $\alpha$ 3, or an  $\alpha$ --subunit receptor as the context will indicate, which retain the biologic and/or immunologic activities of native or naturally-occurring GFR $\alpha$ 3, or receptor. A preferred activity is the ability to bind to and affect, *e.g.*, block or otherwise modulate, an activity of an agonist or natural ligand. The activity preferably involves the regulation of neuronal function.

A "GFR $\alpha$ 3 ligand" is a molecule which binds to and preferably activates native sequence GFR $\alpha$ 3. The ability of a molecule to bind to GFR $\alpha$ 3 can be determined, for example, by the ability of the putative ligand to bind to GFR $\alpha$ 3 immunoadhesin coated on an assay plate, for example. Specificity of binding can be determined by comparing binding to GFR $\alpha$ 1 or 2.

The term "anti-phosphotyrosine antibody" refers to a molecule, usually an antibody, which binds selectively to phosphorylated tyrosine residues in the kinase domain of a rPTK. The antibody can be polyclonal, but is desirably a monoclonal antibody. Anti-phosphotyrosine polyclonal antibodies can be made using the techniques disclosed in White and Backer, Methods in Enzymology 201:65-67 (1991) and  
5 monoclonal anti-phosphotyrosine antibodies can be obtained commercially from Upstate Biologicals, Inc. (UBI, Lake Placid, NY), for example.

The word "label" when used herein refers to a detectable compound or composition which is conjugated directly or indirectly with a molecule (such as the anti-phosphotyrosine antibody). The label may be detectable by itself (*e.g.* radioisotope labels or fluorescent labels) or, in the case of an enzymatic label, may  
10 catalyze a chemical alteration of a substrate compound or composition which is detectable. The preferred label is an enzymatic one which catalyzes a color change of a non-radioactive color reagent.

By "washing" is meant exposing the solid phase to an aqueous solution (usually a buffer or cell culture media) in such a way that unbound material (*e.g.*, non-adhering cells, non-adhering capture agent, unbound ligand, receptor, receptor construct, cell lysate, or anti-phosphotyrosine antibody) is removed therefrom. To  
15 reduce background noise, it is convenient to include a detergent (*e.g.* Triton X) in the washing solution. Usually, the aqueous washing solution is decanted from the wells of the assay plate following washing. Conveniently, washing can be achieved using an automated washing device. Sometimes, several washing steps (*e.g.*, between about 1 to 10 washing steps) may be required.

By "block buffer" is meant an aqueous, pH buffered solution containing at least one blocking  
20 compound which is able to bind to exposed surfaces of the second solid phase which are not coated with capture agent. The blocking compound is normally a protein such as bovine serum albumin (BSA), gelatin, casein or milk powder and does not cross-react with any of the reagents in the assay (*e.g.*, the anti-phosphotyrosine antibodies and detection reagents). The block buffer is generally provided at a pH between about 7 to 7.5 and suitable buffering agents include phosphate and TRIS.

By "lysis buffer" is meant an aqueous, pH buffered solution comprising a solubilizing detergent, one or more protease inhibitors and at least one phosphatase inhibitor (such as sodium orthovanadate). The term  
25 "solubilizing detergent" refers to a water miscible, non-ionic detergent which lyses cell membranes of eukaryotic cells but does not denature or activate the receptor construct. Examples of suitable non-ionic detergents include Triton-X 100, Tween 20, CHAPS and Nonidet P-40 (NP40) available from Calbiochem,  
30 La Jolla, California, for example. Many other non-ionic detergents are available in the art. Examples of suitable protease inhibitors include phenylmethylsulfonyl fluoride (PMSF), leupeptin, pepstatin, aprotinin, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride-bestatin, chymostatin and benzamidine. Preservatives (*e.g.*, thimerosal) and one or more compounds which maintain the isotonicity of the solution (*e.g.*, sodium chloride (NaCl) or sucrose) and a buffer (*e.g.*, Tris or PBS) are usually also present. Generally, the pH of the  
35 lysis buffer is in the range about 7 to 7.5.

Usually, following addition of the lysis buffer to the first assay plate, the first assay plate is "gently agitated" and this expression refers to the act of physically shaking the first assay plate (normally using a circular motion) at a substantially low velocity. Gentle agitation does not involve mechanically disrupting the

cells (e.g. by homogenizing or centrifuging the cells). Exemplary shaking velocities are in the order of 200 to 500 rpm, preferably 300 to 400 rpm in a Bellco orbital shaker, for example.

## II Compositions and Methods of the Invention

### 5 A. Full-length GFR $\alpha$ 3

The present invention provides newly identified and isolated nucleotide sequences encoding polypeptides referred to in the present application as GFR $\alpha$ 3. In particular, Applicants have identified and isolated cDNA encoding a GFR $\alpha$ 3 polypeptide, as disclosed in further detail in the Examples below. Using BLAST, BLAST-2 and FastA sequence alignment computer programs, Applicant has found that a full-length  
 10 native sequence GFR $\alpha$ 3 (SEQ ID NO:15) has 34% amino acid sequence identity with GFR $\alpha$ 1 and GFR $\alpha$ 2. Accordingly, it is presently believed that GFR $\alpha$ 3 disclosed in the present application is a newly identified member of the GFR protein family and may possess neuronal cell activation function typical of the GFR protein family. However, the limited distribution of GFR $\alpha$ 3 compared to GFR $\alpha$ 1 and GFR $\alpha$ 2 make it and its agonists preferred molecules for avoiding unwanted side-effects when administered.

15 Glial cell line-derived neurotrophic factor ("GDNF") and Neurturin ("NTN") are two structurally related, potent survival factors for sympathetic sensory and central nervous system neurons (Lin *et al. Science* 260:1130-1132 (1993); Henderson *et al. Science* 266:1062-1064 (1994); Buj-Bello *et al., Neuron* 15:821-828 (1995); Kotzbauer *et al. Nature* 384:467-470 (1996)). GDNF was shown to mediate its actions through a multi-component receptor system composed of a ligand binding glycosyl-phosphatidyl inositol (GPI) linked  
 20 protein (designated GDNFR $\alpha$  or GFR $\alpha$ 1) and the transmembrane tyrosine kinase Ret (Treanor *et al. Nature* 382:80-83 (1996); Jing *et al. Cell* 85:1113-1124 (1996); Trupp *et al. Nature* 381:785-789 (1996); Durbec *et al. Nature* 381:789-793 (1996)). NTN signal is transmitted by GFR $\alpha$ 2, which also associates with Ret. Described herein is the isolation, sequence, and tissue distribution of a GPI-linked protein and its gene, designated GFR $\alpha$ 3, which is shown to modulate the response to a novel ligand in the NTN and GDNF family.  
 25 In the case of cellular responses to NTN, cells require the presence of GFR $\alpha$ 2. Ligand bound GFR $\alpha$ 2 induces phosphorylation of the tyrosine kinase receptor Ret. These findings identify Ret and GFR $\alpha$ 2, respectively, as signalling and ligand binding components of a receptor for NTN and related ligands. This defines a novel neurotrophic and differentiation factor receptor family of receptors containing a shared transmembrane protein tyrosine kinase (Ret) and a ligand specific GPI-linked protein component (GFR $\alpha$ ).

30 Glial cell line-derived neurotrophic factor ("GDNF") (Lin *et al., Science*, 260:1130-1132 (1993); WO 93/06116, which are incorporated herein in its entirety), is a potent survival factor for midbrain dopaminergic (Lin *et al., Science*, 260:1130-1132 (1993); Strömberg *et al., Exp. Neurol.*, 124:401-412 (1993); Beck *et al., Nature*, 373:339-341 (1995); Kearns *et al., Brain Res.*, 672:104-111 (1995); Tomac *et al., Nature*, 373:335-339 (1995)) spinal motor (Henderson *et al., Science*, 266:1062-1064 (1994); Oppenheim *et al., Nature*, 373:344-346 (1995); Yan *et al., Nature*, 373:341-344 (1995)) and noradrenergic neurons (Arenas *et al., Neuron*, 15:1465-1473 (1995)), which degenerate in Parkinson's disease (Hirsch *et al., Nature*, 334:345-348 (1988); Hornykiewicz *Mt. Sinai J. Med.*, 55:11-20 (1988)), amyotrophic lateral sclerosis (Hirano, Amyotrophic Lateral Sclerosis and Other Motor Neuron Diseases, P. Rowland, ed. (New York: Raven Press,

Inc.) pp. 91-101 (1991)), and Alzheimer's disease (Marcyniuk *et al.*, *J. Neurol. Sci.*, 76:335-345 (1986); Cash *et al.*, *Neurology*, 37:42-46 (1987); Chan-Palay *et al.*, *Comp. Neurol.*, 287:373-392 (1989)) respectively. Based on mice genetically engineered to lack GDNF, additional biological roles for GDNF have been reported: the development and/or survival of enteric, sympathetic, and sensory neurons and the renal system, but not for catecholaminergic neurons in the central nervous system (CNS) (Moore *et al.* *Nature* 382:76-79 (1996); Pichel *et al.* *Nature* 382:73-76 (1996); Sanchez *et al.* *Nature* 382:70-73 (1996)). Despite the physiological and clinical importance of GDNF, little is known about its mechanism of action.

Cytokine receptors frequently assemble into multi-subunit complexes. Sometimes, the  $\alpha$  subunit of this complex is involved in binding the cognate growth factor and the  $\beta$ -subunit may contain an ability to transduce a signal to the cell. Without wishing to be bound by theory, these receptors have been assigned to three subfamilies depending on the complexes formed. Subfamily 1 includes the receptors for EPO, granulocyte colony-stimulating factor (G-CSF), interleukin-4 (IL-4), interleukin-7 (IL-7), growth hormone (GH), and prolactin (PRL). Ligand binding to receptors belonging to this subfamily is thought to result in homodimerization of the receptor. Subfamily 2 includes receptors for IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-5 (IL-5), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (CNTF). Subfamily 2 receptors are heterodimers having an  $\alpha$ -subunit for ligand binding, and  $\beta$ -subunit (either the shared  $\beta$ -subunit of the IL-3, GM-CSF, and IL-5 receptors or the gp130 subunit of the IL-6, LIF, OSM, and CNTF receptors) for signal transduction. Subfamily 3 contains only the interleukin-2 (IL-2) receptor. The  $\beta$  and  $\gamma$  subunits of the IL-2 receptor complex are cytokine-receptor polypeptides which associate with the  $\alpha$ -subunit of the unrelated Tac antigen.

The present invention is based on the discovery of the GFR $\alpha$ 3, a protein in the GFR family, whose natural ligand is unknown. The experiments described herein demonstrate that this molecule is a receptor which appears to play a role in mediating responses to a novel GDNF family ligand. In particular, this receptor has been found to be present in a variety of tissue and cell populations, including neurons, thus indicating that GFR $\alpha$ 3 ligands, such as agonist antibodies, can be used to stimulate proliferation, growth, survival, differentiation, metabolism, or regeneration of GFR $\alpha$ 3- and Ret-containing cells.

#### B. GFR $\alpha$ 3 Variants

In addition to the full-length native sequence GFR $\alpha$ 3 described herein, it is contemplated that GFR $\alpha$ 3 variants can be prepared. GFR $\alpha$ 3 variants can be prepared by introducing appropriate nucleotide changes into the GFR $\alpha$ 3 DNA, or by synthesis of the desired GFR $\alpha$ 3 polypeptides. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the GFR $\alpha$ 3, such as changing the number or position of glycosylation sites or altering the membrane anchoring characteristics. In fact, a splice GFR $\alpha$ 3 splice variant is encoded by DNA48614 and a murine variant by SEQ ID NO: 4. Other variants include the IgG-tagged and gD-RSE chimeras made as described in the Examples.

Variations in the native full-length sequence GFR $\alpha$ 3 or in various domains of the GFR $\alpha$ 3 described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the GFR $\alpha$ 3 that results in a change in the amino acid

sequence of the GFR $\alpha$ 3 as compared with the native sequence GFR $\alpha$ 3. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the GFR $\alpha$ 3. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the GFR $\alpha$ 3 with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity in the *in vitro* assay described in the Examples below.

The variants can be those encoded by an isolated nucleic acid molecule having at least about 65% sequence identity to (a) a nucleic acid sequence encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 27 to 400 of SEQ ID NO: 15, amino acids 27 to 369 of SEQ ID NO: 17 or amino acids 27 to 374 of SEQ ID NO: 5 or (b) the complement of the nucleic acid molecules of (a). Further, the variants can be encoded by nucleic acid molecule sequences comprising a ligand-binding domain of a GFR $\alpha$ 3 polypeptide of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20, or their complementary nucleic acids. These isolated nucleic acid molecules preferably comprise a GFR $\alpha$ 3 encoding sequence which preferably hybridizes under stringent conditions to nucleic acid sequences encoding a GFR $\alpha$ 3 polypeptide of the invention. The sequence identity preferably is at least about 75%, more preferably at least 85%, even more preferably at least 90%, most preferably at least 95%. Typically, the polypeptide has at least about 75%, preferably at least 80%, more preferably at least 85%, even more preferably at least 90%, and most preferably at least about 95% sequence identity with a polypeptide having amino acid residues 27 to 400 of SEQ ID NO: 15, amino acids 27 to 369 of SEQ ID NO: 17, amino acids 27 to 374 of SEQ ID NO: 5, a ligand-binding domain of a GFR $\alpha$ 3 polypeptide of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20. Preferably the identity is to amino acid residues 27 to 400 of SEQ ID NO: 15 and DNA encoding it. The isolated nucleic acid molecule can contain a DNA encoding a GFR $\alpha$ 3 polypeptide having amino acid residues 27 to 400 of SEQ ID NO: 15, or is complementary to such encoding nucleic acid sequence, and remains stably bound to it under at least moderate, and optionally, under high stringency conditions. The protein can be encoded by the nucleic acid encoding the full length protein of clone DNA48613, DNA48614 or murine GFR $\alpha$ 3 (clone 13), deposited with the ATCC under accession numbers ATCC 209752 (Designation: DNA48613-1268), ATCC 209751 (Designation: DNA48614-1268), and ATCC \_\_\_\_\_, respectively, on April 07, 1998, or one that hybridizes thereto under stringent conditions.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis (Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)), cassette mutagenesis (Wells et

al., Gene, 34:315 (1985)), restriction selection mutagenesis (Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)) or other known techniques can be performed on the cloned DNA to produce the GFR $\alpha$ 3 variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions (Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976)). If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used. )

C. Modifications of GFR $\alpha$ 3

Covalent modifications of GFR $\alpha$ 3 are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of the GFR $\alpha$ 3 with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the GFR $\alpha$ 3. Derivatization with bifunctional agents is useful, for instance, for crosslinking GFR $\alpha$ 3 to a water-insoluble support matrix or surface for use in the method for purifying anti-GFR $\alpha$ 3 antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-((p-azidophenyl)dithio)propioimide.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains (T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the GFR $\alpha$ 3 polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence GFR $\alpha$ 3, and/or adding one or more glycosylation sites that are not present in the native sequence GFR $\alpha$ 3, and/or alteration of the ratio and/or composition of the sugar residues attached to the glycosylation site(s).

Addition of glycosylation sites to the GFR $\alpha$ 3 polypeptide may be accomplished by altering the amino acid sequence. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence GFR $\alpha$ 3 (for O-linked glycosylation sites). The GFR $\alpha$ 3 amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the GFR $\alpha$ 3 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the GFR $\alpha$ 3 polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981).

5 Removal of carbohydrate moieties present on the GFR $\alpha$ 3 polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved  
10 by the use of a variety of endo- and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of GFR $\alpha$ 3 comprises linking the GFR $\alpha$ 3 polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417;  
15 4,791,192 or 4,179,337.

The GFR $\alpha$ 3 of the present invention may also be modified in a way to form a chimeric molecule comprising GFR $\alpha$ 3 fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of the GFR $\alpha$ 3 with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or  
20 carboxyl- terminus of the GFR $\alpha$ 3. The presence of such epitope-tagged forms of the GFR $\alpha$ 3 can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the GFR $\alpha$ 3 to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of the GFR $\alpha$ 3 with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric  
25 molecule, such a fusion could be to the Fc region of an IgG molecule.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 (Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)); the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto (Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)); and  
30 the Herpes Simplex virus glycoprotein D (gD) tag and its antibody (Paborsky et al., Protein Engineering, 3(6):547-553 (1990)). Other tag polypeptides include the Flag-peptide (Hopp et al., BioTechnology, 6:1204-1210 (1988)); the KT3 epitope peptide (Martin et al., Science, 255:192-194 (1992)); an  $\alpha$ -tubulin epitope peptide (Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)); and the T7 gene 10 protein peptide tag (Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)).

#### 35 D. Preparation of GFR $\alpha$ 3

The description below relates primarily to production of GFR $\alpha$ 3 by culturing cells transformed or transfected with a vector containing GFR $\alpha$ 3 nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare GFR $\alpha$ 3. For instance, the

GFR $\alpha$ 3 sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques (see, e.g., Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)). *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the GFR $\alpha$ 3 may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the full-length GFR $\alpha$ 3.

1. Isolation of DNA Encoding GFR $\alpha$ 3

DNA encoding GFR $\alpha$ 3 may be obtained from a cDNA library prepared from tissue believed to possess the GFR $\alpha$ 3 mRNA and to express it at a detectable level. Accordingly, human GFR $\alpha$ 3 DNA can be conveniently obtained from a cDNA library prepared from human tissue, such as described in the Examples. The GFR $\alpha$ 3 -encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the GFR $\alpha$ 3 or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding GFR $\alpha$ 3 is to use PCR methodology (Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)).

The Examples below describe techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like  $^{32}\text{P}$ -labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined through sequence alignment using computer software programs such as BLAST, BLAST-2, ALIGN, DNASTar, and INHERIT which employ various algorithms to measure homology.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

2. Selection and Transformation of Host Cells



Host cells are transfected or transformed with expression or cloning vectors described herein for GFR $\alpha$ 3 production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: a Practical Approach, M. Butler, ed. (IRL Press, 1991) and Sambrook et al., supra.

Methods of transfection are known to the ordinarily skilled artisan, for example, CaPO<sub>4</sub> and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook *et al.*, supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., Gene, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology, 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen et al., J. Bact., 130:946 (1977) and Hsiao et al., Proc. Natl. Acad. Sci. (USA), 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown et al., Methods in Enzymology, 185:527-537 (1990) and Mansour et al., Nature, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriaceae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635).

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for GFR $\alpha$ 3 -encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Suitable host cells for the expression of glycosylated GFR $\alpha$ 3 are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as *Drosophila* S2 and *Spodoptera* Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham et al., J. Gen. Virol., 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, Biol. Reprod., 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

### 3. Selection and Use of a Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding GFR $\alpha$ 3 may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques which are known to the skilled artisan.

The GFR $\alpha$ 3 may be produced recombinantly not only directly, but also as a fusion polypeptide with a heterologous polypeptide, which may be a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the GFR $\alpha$ 3 DNA that is inserted into the vector. The signal sequence may be a prokaryotic signal sequence selected, for example, from the group of the alkaline phosphatase, penicillinase, lpp, or heat-stable enterotoxin II leaders. For yeast secretion the signal sequence may be, *e.g.*, the yeast invertase leader, alpha factor leader (including *Saccharomyces* and *Kluyveromyces*  $\alpha$ -factor leaders, the latter described in U.S. Patent No. 5,010,182), or acid phosphatase leader, the *C. albicans* glucoamylase leader (EP 362,179 published 4 April 1990), or the signal described in WO 90/13646 published 15 November 1990. In mammalian cell expression, mammalian signal sequences may be used to direct secretion of the protein, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2: plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the GFR $\alpha$ 3 nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub et al., Proc. Natl. Acad. Sci. USA, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp1* gene present in the yeast plasmid YRp7 (Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)). The *trp1* gene provides a

selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)).

Expression and cloning vectors usually contain a promoter operably linked to the GFR $\alpha$ 3 nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known.

5 Promoters suitable for use with prokaryotic hosts include the  $\beta$ -lactamase and lactose promoter systems (Chang et al., Nature, 275:615 (1978); Goeddel et al., Nature, 281:544 (1979)), alkaline phosphatase, a tryptophan (trp) promoter system (Goeddel, Nucleic Acids Res., 8:4057 (1980); EP 36,776), and hybrid promoters such as the tac promoter (deBoer et al., Proc. Natl. Acad. Sci. USA, 80:21-25 (1983)). Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA

10 encoding GFR $\alpha$ 3.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem., 255:2073 (1980)) or other glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg., 7:149 (1968); Holland, Biochemistry, 17:4900 (1978)), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase,

15 glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors

20 and promoters for use in yeast expression are further described in EP 73,657.

GFR $\alpha$ 3 transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a

25 retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a DNA encoding the GFR $\alpha$ 3 by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300

30 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin,  $\alpha$ -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at

35 a position 5' or 3' to the GFR $\alpha$ 3 coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and,

occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding GFR $\alpha$ 3. Still other methods, vectors, and host cells suitable for adaptation to the synthesis of GFR $\alpha$ 3 in recombinant vertebrate cell culture are described in Gething et al., Nature, 293:620-625 (1981); Mantei et al., Nature, 281:40-46 (1979); EP 117,060; and EP 117,058.

4. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA (Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)), dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence GFR $\alpha$ 3 polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to GFR $\alpha$ 3 DNA and encoding a specific antibody epitope.

5. Purification of Polypeptide

Forms of GFR $\alpha$ 3 may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or by enzymatic cleavage. Cells employed in expression of GFR $\alpha$ 3 can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents. It may be desired to purify GFR $\alpha$ 3 from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the GFR $\alpha$ 3. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular GFR $\alpha$ 3 produced.

E. Uses for GFR $\alpha$ 3

Nucleotide sequences (or their complement) encoding GFR $\alpha$ 3 have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome and gene mapping and in the generation of anti-sense RNA and DNA. GFR $\alpha$ 3 nucleic acid will also be useful for the preparation of GFR $\alpha$ 3 polypeptides by the recombinant techniques described herein.

5       The full-length native sequence GFR $\alpha$ 3 (in SEQ ID NO:14) gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate the full-length gene or to isolate still other genes (for instance, those encoding naturally-occurring variants of GFR $\alpha$ 3 or GFR $\alpha$ 3 from other species) which have a desired sequence identity to the GFR $\alpha$ 3 sequence disclosed in SEQ ID NO: 15. Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from the nucleotide  
10       sequence of SEQ ID NO:14 or from genomic sequences including promoters, enhancer elements and introns of native sequence GFR $\alpha$ 3. By way of example, a screening method will comprise isolating the coding region of the GFR $\alpha$ 3 gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as <sup>32</sup>P or <sup>35</sup>S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems.  
15       Labeled probes having a sequence complementary to that of the GFR $\alpha$ 3 gene of the present invention can be used to screen libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes to. Hybridization techniques are described in further detail in the Examples below.

20       The probes may also be employed in PCR techniques to generate a pool of sequences for identification of closely related GFR $\alpha$ 3 sequences.

      Nucleotide sequences encoding a GFR $\alpha$ 3 can also be used to construct hybridization probes for mapping the gene which encodes that GFR $\alpha$ 3 and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosome using known techniques, such as *in situ* hybridization, linkage analysis against known  
25       chromosomal markers, and hybridization screening with libraries.

      When the coding sequences for GFR $\alpha$ 3 encode a protein which binds to another protein (example, where the GFR $\alpha$ 3 is a receptor), the GFR $\alpha$ 3 can be used in assays to identify the other proteins or molecules involved in the binding interaction. By such methods, inhibitors of the receptor/ligand binding interaction can be identified. Proteins involved in such binding interactions can also be used to screen for peptide or small  
30       molecule inhibitors or agonists of the binding interaction. Also, the receptor GFR $\alpha$ 3 can be used to isolate correlative ligand(s). Screening assays can be designed to find lead compounds that mimic the biological activity of a native GFR $\alpha$ 3 or a receptor for GFR $\alpha$ 3. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds.  
35       The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

      Nucleic acids which encode GFR $\alpha$ 3 or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of

therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA which is integrated into the genome of a cell from which a transgenic animal develops. In one embodiment, cDNA encoding GFR $\alpha$ 3 can be used to clone genomic DNA encoding GFR $\alpha$ 3 in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding GFR $\alpha$ 3. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for GFR $\alpha$ 3 transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding GFR $\alpha$ 3 introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding GFR $\alpha$ 3. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Non-human homologues of GFR $\alpha$ 3 can be used to construct a GFR $\alpha$ 3 "knock out" animal which has a defective or altered gene encoding GFR $\alpha$ 3 as a result of homologous recombination between the endogenous gene encoding GFR $\alpha$ 3 and altered genomic DNA encoding GFR $\alpha$ 3 introduced into an embryonic cell of the animal. For example, cDNA encoding GFR $\alpha$ 3 can be used to clone genomic DNA encoding GFR $\alpha$ 3 in accordance with established techniques. A portion of the genomic DNA encoding GFR $\alpha$ 3 can be deleted or replaced with another gene, such as a gene encoding a selectable marker which can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector (see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors). The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected (see e.g., Li et al., Cell, 69:915 (1992)). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras (see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the GFR $\alpha$ 3 polypeptide.

Agents which bind to the GFR $\alpha$ 3 molecule could be useful in the treatment of diseases or conditions involving the peripheral nervous system. For example, such ligands can be used to treat peripheral neuropathies associated with diabetes, HIV, chemotherapeutic agent treatments. Ligands binding to GFR $\alpha$ 3 are expected to be useful in the treatment of neuropathic pain, antagonists of GFR $\alpha$ 3 are expected to be useful

to treat chronic pain of non-neuropathic nature such as, but not limited to, that which is associated with various inflammatory states. The above therapies are consistent with the data of Example 5 in which a strong expression of GFR $\alpha$ 3 within developing and adult sensory ganglia was observed. GFR $\alpha$ 3 or its agonist or antagonists can be used to treat conditions involving dysfunction of the autonomic nervous system including, but not limited to, disturbances in blood pressure or cardiac rhythm, gastrointestinal function, impotence, and urinary continence. Other indications for ligands binding to GFR $\alpha$ 3 include: post-herpetic neuralgia, shingles, asthma, irritable bowel, inflammatory bowel, cystitis, headache (migraine), arthritis, spinal cord injury, constipation, hypertension, mucositis, dry mouth or eyes, fibromyalgia, chronic back pain, or wound healing. These uses are consistent with the observed expression in sympathetic ganglia.

The surprising, relative lack of expression of GFR $\alpha$ 3 in many organs, including notably brain, gut, and kidney indicates that the ligand (and other agonists or antagonists) which binds this receptor lacks some side effects which may be associated with ligands which bind to GFR $\alpha$ 1 and GFR $\alpha$ 2 (GDNF and neurturin). Thus, ligands which act via GFR $\alpha$ 3 will be particularly useful to treat disorders of the peripheral nervous system while inducing fewer effects on weight loss, motor function, or on kidney function than would ligands acting via GFR $\alpha$ 1 or GFR $\alpha$ 2.

F. Anti-GFR $\alpha$ 3 Antibodies

The present invention further provides anti-GFR $\alpha$ 3 antibodies. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The anti-GFR $\alpha$ 3 antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the GFR $\alpha$ 3 polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants which may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be selected by one skilled in the art without undue experimentation.

2. Monoclonal Antibodies

The anti-GFR $\alpha$ 3 antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the GFR $\alpha$ 3 polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103). Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al. Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63).

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against GFR $\alpha$ 3. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, supra). Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression



vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences (U.S. Patent No. 4,816,567; Morrison et al., *supra*) or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

### 3. Humanized Antibodies

The anti-GFR $\alpha$ 3 antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')<sub>2</sub> or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin (Jones et al., *Nature*, 321:522-525 (1986); Riechmann et al., *Nature*, 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.*, 2:593-596 (1992)).

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and

co-workers (Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries (Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)). The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)).

#### 4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the GFR $\alpha$ 3, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities (Milstein and Cuello, Nature, 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are co-transfected into a suitable host organism. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

#### 5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Patent No. 4,676,980), and for treatment of HIV infection (WO 91/00360; WO 92/200373; EP 03089). It is contemplated that the antibodies

may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

5           G.       Uses for anti-GFR $\alpha$ 3 Antibodies

The anti-GFR $\alpha$ 3 antibodies of the invention have various utilities. For example, anti-GFR $\alpha$ 3 antibodies may be used in diagnostic assays for GFR $\alpha$ 3, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases (Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158). The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{32}\text{P}$ ,  $^{35}\text{S}$ , or  $^{125}\text{I}$ , a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David et al., Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 32:407 (1982).

Anti-GFR $\alpha$ 3 antibodies also are useful for the affinity purification of GFR $\alpha$ 3 from recombinant cell culture or natural sources. In this process, the antibodies against GFR $\alpha$ 3 are immobilized on a suitable support, such as a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the GFR $\alpha$ 3 to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the GFR $\alpha$ 3, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the GFR $\alpha$ 3 from the antibody.

25           H.       Assays For Ligand-induced A-Subunit Activity

The compounds and methods of the invention can be used in assays to detect molecules that activate or inhibit GFR $\alpha$ 3 signal transduction, and indeed can be applied to other  $\alpha$ -subunit receptor molecules (*e.g.*, GFR $\alpha$ 1, GFR $\alpha$ 2, GFR $\alpha$ 4) that homodimerize or homo-oligomerize upon activation by a ligand or other agonist. The assays are based on the surprising fact that the  $\alpha$ -subunit receptors can homo-dimerize or homo-oligomerize upon ligand binding. And further that this dimerization of an  $\alpha$ -subunit, when fused to a receptor protein kinase intracellular domain capable of kinase activity, preferably tyrosine kinase activity, results in kinase activity, *e.g.* readily detectable autophosphorylation. While the methods and constructs herein are discussed in terms of one or another GFR $\alpha$  subunit receptor disclosed herein, the methods will apply readily to any  $\alpha$ -receptor in the  $\alpha$ -subunit receptor family--a family in which the  $\alpha$ -subunit receptor is the ligand-binding partner of a multi-subunit signal transduction complex containing a beta subunit that typically contains a tyrosine kinase activity that is activated upon ligand-activated  $\alpha$ -subunit binding to the beta subunit.

Various assays have been developed which measure kinase activity, and in particular tyrosine kinase activity. Some of these assays measure the ability of a tyrosine kinase enzyme to phosphorylate a synthetic substrate polypeptide. For example, an assay has been developed which measures growth factor-stimulated tyrosine kinase activity by measuring the ability of the kinase to catalyze the transfer of the  $\gamma$ -phosphate of ATP to a suitable acceptor substrate. See Pike, L., Methods of Enzymology **146**:353-362 (1987) and Hunter, Journal of Biological Chemistry **257**(9):4843-4848 (1982), for example. In this assay, the use of ( $\gamma$ - $^{32}\text{P}$ )ATP permits the radioactive labeling of the phosphorylated substrate, which is a synthetic tyrosine-containing peptide. Others have described protein kinase assays wherein incorporation of  $^{32}\text{P}$  into a tyrosine kinase receptor, such as the EGF receptor (see Donato *et al.*, Cell Growth Differ. **3**:259-268 (1992)), insulin receptor (see Kasuga *et al.*, Journal of Biological Chemistry **257**(17):9891-9884 (1982) and Kasuga *et al.*, Methods in Enzymology **109**:609-621 (1985)), and liver growth hormone receptor (see Wang *et al.*, Journal of Biological Chemistry **267**(24):17390-17396 (1992)), is measured.

Construction of  $\alpha$ -receptor constructs, including fusions to Rse or other tyrosine kinase domains, vectors for expressing such constructs, transfected or transformed host cells expressing these constructs, and means to enhance their expression at the cell surface are achieved as would be known in the art using, for example, the techniques as described herein for GFR $\alpha$ 3 expression. Some particularly preferred means are provided below.

#### 1. Kinase Receptor Activation - K $\alpha$ RA

The first stage of an assay of the invention involves phosphorylation of the kinase domain of a receptor construct, wherein the receptor construct is present in the cell membrane of a eukaryotic cell. The receptor construct can be derived from a nucleic acid encoding the receptor construct (as described herein) that can be transformed into the cell. In one embodiment of the invention, nucleic acid encoding a receptor construct is transformed into the cell. Preferred and exemplary techniques for transforming the cell with either the receptor or the receptor construct nucleic acid follow.

##### 25 a. Transformation of the cells

The instant invention provides a substantial improvement over in vitro soluble kinase receptor assays insofar as it is considered to more accurately reflect the activity of the  $\alpha$ --subunit receptor *in situ*. It has been discovered that it is possible to transform eukaryotic cells with a receptor construct (comprising the  $\alpha$ --subunit receptor and a kinase domain fusion and optionally, either an amino- or carboxyl-terminal flag polypeptide) so that the receptor construct assembles itself appropriately in the cell membrane and still retains kinase activity which can be detected in the ELISA stage of the assay. This provides a generic assay for measuring ligand binding activity, via the kinase activity of the fusion, of any  $\alpha$ --subunit receptor of interest that homo-dimerizes or homo-oligomerizes upon ligand-binding.

If a suitable capture agent as described herein is available for a selected receptor construct, cells can be transformed with the nucleic acid encoding the receptor construct alone, without the flag polypeptide.

In order to provide nucleic acid encoding a receptor construct, nucleic acid encoding the  $\alpha$ --subunit receptor is fused at its 3' end to nucleic acid encoding the intracellular catalytic kinase domain of a receptor kinase, preferably a rPTK, including a transmembrane domain, and optionally to the the N-terminus of the flag

polypeptide. Alternatively, the nucleic acid encoding the  $\alpha$ -subunit receptor-kinase domain fusion will be fused at its 5' end to nucleic acid encoding the carboxyl terminus of the flag polypeptide. Thus, the flag polypeptide is provided at either the carboxyl- or amino- terminus of the receptor construct. Examples of suitable flag polypeptides are provided above. Selection of other suitable flag polypeptides is possible using the techniques described herein.

In order to generate fusions between the Rse.flag reagent and a  $\alpha$ -subunit receptor of interest, the nucleic acid encoding the ECD (or GPI-anchor minus variant) of the  $\alpha$ -subunit receptor of interest is fused at its 3' end to the nucleic acid encoding the amino terminus of the Rse.flag reagent.

Incorporation of a signal sequence into the expression vector is required since the receptor construct must be transported to the cell membrane where it is positioned such that the ECD faces the external milieu of the cell. Therefore, a signal sequence suitable for positioning the receptor construct in such a manner is used. The signal sequence is generally a component of the vector, or it may be a part of the receptor construct DNA that is inserted into the vector. If a heterologous signal sequence is used, it is from those that are recognized and processed (*i.e.*, cleaved by a signal peptidase) by the host cell.

*b. Selecting cells for use in the assay*

As mentioned above, the cells to be subjected to the assay are preferably cells transformed with a receptor construct. The suitability of the cells for use in the assay is investigated.

If the cell line is transformed with the receptor construct (without the flag polypeptide) it can be readily discovered whether the cell line is suitable for use in the assay. As a first step, successful transformation and expression of the nucleic acid encoding the receptor construct is determined. The strategy found in U.S. Patent 5,766,863, or its corresponding WO publication, entitled "Kinase receptor activation assay" can be followed. In order to identify whether the ECD of the receptor construct is present on the surface of the cells, flow cytometric analysis can be performed using an antibody to the ECD of the  $\alpha$ -subunit receptor. The antibody can be made using the techniques for generating antibodies discussed herein. Flow cytometric analysis can be carried out using the techniques described in Current Protocols in Immunology, Ed. Coligen *et al.*, Wiley publishers, Vols. 1 and 2, for example. Briefly, flow cytometric analysis involves incubating intact cells (having the receptor) with antibodies to the ECD thereof, followed by washing. The antibody-bound cells are then incubated with species specific anti-antibody antibodies conjugated to a fluorochrome. Following washing, the labeled cells are analyzed by fluorescence-activated flow cytometry to detect whether the ECD is present on the surface of the cells.

In the following step, the ability of the cell-bound receptor to be activated is tested. In order to determine this, the transformed cells are exposed to a known agonist to the receptor (*e.g.* the endogenous ligand or an agonist antibody for the receptor). In the case of GFR $\alpha$ 3 the natural ligand is artemin. Following exposure, the cells are lysed in a suitable buffer (*e.g.* sodium dodecylbenzenesulfonate in phosphate buffered saline; SDS in PBS) and subjected to Western blotting with anti-phosphotyrosine antibodies as described in Wang, Molecular and Cellular Biology 5(12):3640-3643 (1985); Glenney *et al.*, Journal of Immunological Methods 109:277-285 (1988); Kamps, Methods in Enzymology 201:101-110 (1991); Kozma *et al.*, Methods

in Enzymology 201:28-43 (1991); Holmes *et al.*, Science 256:1205-10 (1992); or Corfas *et al.*, PNAS, USA 90:1624-1628 (1993), for example.

Assuming the Western blotting step indicates that the receptor construct can be activated, a KIRA ELISA test run can be performed to further establish whether or not the transformed cell line can be used in the assay.

In the preferred embodiment of the invention, the KIRA ELISA is a "generic" assay insofar as any  $\alpha$ --subunit receptor of interest can be studied regardless of the availability of receptor-specific reagents (*i.e.*, capture agent). This embodiment employs a receptor construct having a flag polypeptide at either the amino or carboxyl terminus of the receptor.

If the flag polypeptide is provided at the NH<sub>2</sub>-terminus (see, *e.g.*, the gD.trk A, B and C receptor constructs), the procedure for selecting a transformed cell line for use in the assay is similar to that described above. In this embodiment, the cells are transformed with the flag polypeptide-receptor construct as described earlier herein. Successful transformation of the receptor and flag polypeptide (*i.e.* the receptor construct in this example) is confirmed. In order to study this, two-dimensional flow cytometric analysis can be performed using antibodies to both the flag polypeptide and the ECD of the receptor. Techniques for two-dimensional flow cytometric analysis are disclosed in Current Protocols in Immunology, *supra*.

Cells which have been successfully transformed with the receptor construct having a C-terminal flag polypeptide are also suitable for use in the assay. Following cell transformation, successful transformation of the receptor is determined by flow cytometric analysis using an antibody directed against the ECD of the receptor of interest, for example. Flow cytometric analysis can be performed substantially as described above.

Successful transformation of the entire receptor construct (including the COOH-terminal flag polypeptide) is then analyzed. This can be achieved by lysing the cells (using techniques for lysing cells disclosed herein) and immunoprecipitating the membrane extract with an antibody against the  $\alpha$ --subunit receptor of interest. This immunoprecipitated membrane extract is then subjected to Western blot analysis with antibodies specific for the flag polypeptide. Alternatively,  $\alpha$ --subunit specific ELISA analysis of anti-flag polypeptide captured membrane lysate can be carried out. Briefly, this involves coating ELISA wells with appropriate flag specific capture agent. The wells are blocked, washed, and the lysate is then incubated in the wells. Unbound receptor construct is removed by washing. The wells are then reacted with receptor-specific antibody or antibodies, either directly or indirectly conjugated to HRPO. The wells are washed and the HRPO is then exposed to the chromogenic substrate (*e.g.*, TMB). Detecting receptor activation and KIRA ELISA test run, are essentially the same as those steps described above.

Once useful cells are identified, they are subjected to the KIRA stage of the instantly claimed assay.

*c. Coating the first solid phase with the cells*

The first solid phase (*e.g.* a well of a first assay plate) is coated with cells which have been transformed pursuant to the preceding sections.

Preferably, an adherent cell line is chosen, so that the cells naturally adhere to the first solid phase. However, use of an adherent cell line is not essential. For example, non-adherent cells (*e.g.* red blood cells) can be added to round bottomed wells of an assay plate such as that sold by Becton Dickinson Labware,

Lincoln Park, New Jersey, for example. The assay plate is then placed in a plate carrier and centrifuged so as to create a pellet of cells adhering to the base of the wells. The cell culture supernatants are removed using a pipette. Thus, use of an adherent cell is clearly advantageous over non-adherent cells since it reduces variability in the assay (*i.e.*, the cells in the pellet of the round bottom wells may be taken up with the supernatant when the alternative method is used).

The cells to be added to the wells of the first assay plate may be maintained in tissue culture flasks and utilized when cells densities of about 70-90% confluency are achieved. Then, generally between about  $1 \times 10^4$  to  $3 \times 10^5$  (and preferably  $5 \times 10^4$  to  $1 \times 10^5$ ) cells are seeded per flat-bottom well, using a pipette, for example. It has been found that, contrary to expectations, addition of cell concentrations mentioned above is sufficient to enable activation of the receptor construct to be measured in the ELISA stage of the assay, without the need to concentrate or clarify the cells or cell lysate prior thereto. Often, the cells are diluted in culture medium prior to seeding them in the wells of the microtiter plate to achieve the desired cell densities.

Usually, the cells are cultured in the microtiter plates for a sufficient period of time to optimize adherence to the wells thereof, but not too long such that the cells begin to deteriorate. Thus, incubation for about 8 to 16 hours at a temperature which is the physiological optimum for the cells (usually about 37°C) is preferred. Suitable media for culturing the cells are described in Section 1A above. Culturing in 5% CO<sub>2</sub> is recommended.

Following incubation overnight, the well supernatants are decanted and excess supernatant may be further removed by lightly tamping the microtiter plates with an absorbent substrate, *e.g.*, a paper towel, but a sponge works equally well. Thus, a substantially homogeneous layer of adhering cells remains on the internal surfaces of the individual wells of the microtiter plate. These adhering cells are then exposed to the analyte.

*d. Preparation and addition of the analyte*

As mentioned above, the analyte may comprise an agonist ligand (or suspected agonist) or an antagonist (or suspected antagonist) for the  $\alpha$ -subunit receptor of interest. The ligand may be an endogenous polypeptide, or a synthetic molecule, such as an inorganic or organic molecule. Usually, the ligand is a polypeptide. This assay is useful for screening molecules which activate (or antagonize activation) of the  $\alpha$ -subunit receptor of interest. Thus, the assay can be used for developing therapeutically effective molecules.

Where the ligand is an agonist, the molecule can comprise the native growth factor *e.g.*, artemin, neurturin, GDNF, and persephin. Many of these growth factors are available commercially. Alternatively, the growth factor can be made by peptide synthesis or recombinant techniques which are described herein. Synthetic small molecule agonists can similarly be generated by those skilled in the art using conventional chemical synthesis techniques. Preferably, one is assaying for agonist or antagonist antibodies.

Where the ligand is present in a biological fluid, the analyte can be prepared using techniques which are well known in the art. Body fluid such as blood or amniotic fluid may be used directly, however concentration may be required. If the analyte to be tested comprises a particular tissue, the cells thereof can be grown in cell culture and the supernatant can be tested for secreted ligand.

Often, the ligand is diluted in an aqueous diluent (such as cell culture media) so that a standard curve can be generated. However, the ligand may be present in a cell or a cell component (*e.g.*, the cell membrane). In particular, it has been found that the assay can be used to detect the presence of a ligand in the cell membrane of a selected cell line. This is clearly useful for discovering a novel endogenous ligand for a known  $\alpha$ -subunit receptor.

The ligand composition is added to each well which contains the adhering cells using a pipette, for example. At least one control well (*e.g.* to which the aqueous diluent for the ligand is added) is included in the assay.

The adhering cells are usually stimulated for a sufficient period of time to optimize the signal, but not too long such that the signal decreases as a consequence of dephosphorylation of the receptor by endogenous phosphatases. A suitable stimulation period is between about 10 to 60 minutes, preferably about 30 minutes at a physiologically optimal temperature for the cells (usually about 37°C).

Following activation, well supernatants are decanted and the plates can then be lightly tamped with an absorbent substrate to remove excess supernatant.

The assay can be used to detect antagonist ligands for the receptor of interest. Antagonists generally fall into two categories (a) ones which bind to the receptor and thereby block binding and/or activation of the receptor by an agonist thereto (the antagonist may bind to the ECD, but this is not necessarily the case) and (b) those which bind to the agonist and thus prevent activation of the receptor by the agonist.

In order to detect antagonist molecules from category (a) above, the cells are exposed to the suspected antagonist ligand substantially as mentioned above. Following exposure to the antagonist, the well supernatants are decanted and the plates are lightly tamped. Then, a known agonist (*e.g.*, the endogenous growth factor) is added to the washed cells essentially as discussed in the preceding paragraphs, following which, the well supernatants are decanted and plates are lightly tamped. Alternatively, a composition comprising both the antagonist and agonist can be added to the adhering cells substantially as discussed above. Ability of the suspected antagonist to block binding and/or activation of the receptor can subsequently be measured by ELISA as discussed below.

To detect antagonist molecules from category (b) above, a known agonist is pre-incubated with the suspected antagonist prior to the KIRA stage of the assay. This incubation is carried out for a sufficient period of time to enable a complex of the antagonist-agonist to form; from 30 min. to 12 hours, for example. This complex is then subjected to the assay with the non-complexed agonist and antagonist used as controls.

Following exposure to the agonist (and optionally the antagonist) ligand, the cells are lysed, as discussed below.

*e. Solubilizing the cells*

In this step of the assay, the cells are lysed so as to solubilize the receptor construct such that it remains activated (*i.e.*, the tyrosine residues remain phosphorylated) for the ELISA stage of the assay. Thus, the cells are lysed using a lysis buffer as described above which serves to solubilize the receptor construct, yet does not dephosphorylate or denature the receptor construct.



Where microtiter plates are used as mentioned above, about 75 to 200 $\mu$ l of lysis buffer is added to each well. The plates can then be agitated gently using a plate shaker (*e.g.*, such as that sold by Bellco Instruments, Vineland, NJ) for about 1 to 2 hours. Shaking can be carried out at room temperature.

2. Enzyme-Linked Immunosorbent Assay - ELISA

5 The second stage of the assay involves a sandwich ELISA performed in the second assay plate. In order to carry out the ELISA, a capture agent is prepared.

a. *Preparation of the capture agent*

As mentioned above, the capture agent often comprises a polyclonal antibody (usually an affinity purified polyclonal antibody) or monoclonal antibody. Other capture agents are  
10 envisaged and are discussed in the definitions section above. The capture agent either binds specifically to the receptor, or to the flag polypeptide (*i.e.* the antigen).

Polyclonal antibodies to the antigen (either the receptor or the flag polypeptide) generally are raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the antigen or an antigenic fragment thereof (often the ECD of the  $\alpha$ -subunit receptor) and an adjuvant. It may be useful to conjugate  
15 the antigen or a fragment containing the target amino acid sequence to a protein that is immunogenic in the species to be immunized (*e.g.*, keyhole limpet hemocyanin), using a bifunctional or derivatizing agent.

The route and schedule for administration of immunogen to the host animal or cultured antibody-producing cells therefrom are generally in keeping with established and conventional techniques for antibody stimulation and production. While mice are frequently employed as the test model, it is contemplated that any  
20 mammalian subject including human subjects or antibody-producing cells obtained therefrom can be manipulated according to the processes of this invention to serve as the basis for production of mammalian, including human, hybrid cell lines.

Animals are typically immunized against the immunogenic conjugates or derivatives by combining 1 mg or 1  $\mu$ g of conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with 1/5  
25 to 1/10 the original amount of conjugate in Freund's complete adjuvant (or other suitable adjuvant) by subcutaneous injection at multiple sites. 7 to 14 days later animals are bled and the serum is assayed for anti-antigen titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking agent.  
30 Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are used to enhance the immune response.

After immunization, monoclonal antibodies can be prepared by recovering immune cells (typically spleen cells or lymphocytes from lymph node tissue) from immunized animals and immortalizing the cells in conventional fashion, *e.g.*, by fusion with myeloma cells or by Epstein-Barr (EB)-virus transformation and  
35 screening for clones producing the desired antibody. The hybridoma technique described originally by Kohler and Milstein, Eur. J. Immunol. 6:511 (1976), and also described by Hammerling *et al.*, In: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981) has been widely applied to produce hybrid cell lines that secrete high levels of monoclonal antibodies against many specific antigens.

It is possible to fuse cells of one species with another. However, it is preferable that the source of the immunized antibody producing cells and the myeloma be from the same species.

The hybrid cell lines can be maintained in culture in cell culture media. The cell lines of this invention can be selected and/or maintained in a composition comprising the continuous cell line in hypoxanthine-aminopterin-thymidine (HAT) medium. In fact, once the hybridoma cell line is established, it can be maintained on a variety of nutritionally adequate media. Moreover, the hybrid cell lines can be stored and preserved in any number of conventional ways, including freezing and storage under liquid nitrogen. Frozen cell lines can be revived and cultured indefinitely with resumed synthesis and secretion of monoclonal antibody.

The secreted antibody is recovered from tissue culture supernatant by conventional methods such as precipitation, ion exchange chromatography, affinity chromatography, or the like. The antibodies described herein are also recovered from hybridoma cell cultures by conventional methods for purification of IgG or IgM, as the case may be, that heretofore have been used to purify these immunoglobulins from pooled plasma, *e.g.*, ethanol or polyethylene glycol precipitation procedures. The purified antibodies are then sterile filtered. Where the antibody is a polyclonal antibody, it is generally affinity purified using an affinity column generated from the antigen of interest so as to provide a substantially specific capture antibody. Affinity chromatography is usually preceded by other purification techniques, such as liquid chromatography.

In a further embodiment, antibodies or antibody fragments can be isolated from antibody phage libraries generated via the techniques described in McCafferty *et al.*, Nature, **348**:552-554 (1990), using the flag polypeptide,  $\alpha$ -subunit receptor, or a fragment thereof, to select for a suitable antibody or antibody fragment. Clackson *et al.*, Nature, **352**:624-628 (1991) and Marks *et al.*, J. Mol. Biol., **222**:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Mark *et al.*, Bio/Technol. **10**:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, Nuc. Acids Res., **21**:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional monoclonal antibody hybridoma techniques for isolation of "monoclonal" antibodies which are encompassed by the present invention.

DNA encoding the monoclonal antibodies of the invention is readily isolated and sequenced using conventional procedures (*e.g.*, by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences, Morrison *et al.*, Proc. Nat. Acad. Sci. **81**, 6851 (1984), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. In that manner, "chimeric" or "hybrid" antibodies are prepared that have the binding specificity of an anti-receptor or anti-flag polypeptide

monoclonal antibody herein. Thus, the antibody may be made by recombinant DNA methods (Cabilly *et al.*, U.S. Pat. No. 4,816,567).

Binding of the capture agent is not affected by the presence or absence of a ligand bound to the receptor and the capture agent does not sterically block access to the phosphorylated tyrosine(s) by the anti-phosphotyrosine antibody. Furthermore, the capture agent does not, of course, activate the receptor of interest.

First, once the capture agent (*e.g.* an antibody or streptavidin) has been chosen, binding to either the receptor or the flag polypeptide (where a receptor construct is to be used in the assay) is confirmed. This can be determined by flow cytometric analysis, immuno-precipitation or antigen-coat ELISA, for example. Flow cytometric analysis has been described above. Immunoprecipitation usually involves lysing the cells (having the receptor construct) in non-ionic detergent (*e.g.* 0.5% Triton X-100) in a suitable buffer (*e.g.* PBS) and the cell lysates thus obtained are then incubated with the potential anti-receptor or anti-flag polypeptide capture agent. The immune complexes are precipitated with either (a) anti-capture agent antibodies in the presence of polyethylene glycol (PEG) which enhances precipitation of the immune complex or with (b) insoluble (*e.g.* agarose bound) protein A or protein G. The immunoprecipitated material is then analyzed by polyacrylamide gel electrophoresis (PAGE). For antigen-coat ELISA, ELISA wells are coated overnight with either the purified receptor, purified flag polypeptide or purified receptor construct. The coated wells are then exposed to the potential capture agent and screened with HRPO-conjugated species specific anti-capture agent antibody.

The ability of the capture agent to bind to the receptor or flag polypeptide in the presence of a ligand to the receptor is also confirmed. This can be analyzed by incubating the receptor construct with a known ligand for the receptor (*e.g.* the endogenous growth factor or an agonist antibody thereto). Flow cytometric analysis, immunoprecipitation or antigen-coat ELISA can then be performed substantially as described above to investigate binding of the capture agent.

Assuming the capture agent is suitable as determined by the preceding two steps, it is then shown that the capture agent does not induce receptor activation (*i.e.* autophosphorylation) either before or after cell lysis. Thus, the cell-bound receptor construct is exposed to either the potential capture agent or a negative control (*e.g.* a control antibody which does not activate the receptor). Following cell lysis, the receptor construct can be subjected to Western blot analysis using labeled anti-phosphotyrosine antibodies. See, *e.g.*, Glenney *et al.*, Journal of Immunological Methods **109**:277-285 (1988); Kamps, Methods in Enzymology **201**:101-110 (1991); Kozma *et al.*, Methods in Enzymology **201**:28-43 (1991); or Holmes *et al.*, Science **256**:1205-10 (1992). To establish whether the capture agent induces receptor activation following cell lysis, a trial run of the KIRA ELISA (with both the capture agent and a negative control as discussed above) can be performed.

Finally, the ability of an anti-phosphotyrosine antibody (*e.g.* biotinylated anti-phosphotyrosine antibody) to bind the activated receptor in the presence of the potential capture agent is confirmed by a trial run in the KIRA ELISA disclosed herein.

Assuming the capture agent meets all the criteria specified above, it has good potential for use in the KIRA ELISA.

Once a suitable capture agent has been prepared, the second solid phase is coated therewith. Between about 0.1 to 10 µg/ml of capture agent can be added to each well of the second assay plate using a pipette, for example. The capture agent is often provided in a buffer at a high pH (*e.g.*, between about 7.5 to 9.6) so that it has an increased overall charge and therefore displays enhanced binding to the second assay plate. Usually, the capture agent will be incubated in the wells for between about 8 to 72 hours to enable a sufficient coating of the capture agent to form on the inside walls of the wells. This incubation is generally carried out at low temperatures (*e.g.*, between about 3-8°C) to avoid or reduce degradation of the capture agent.

Following incubation, the wells of the plate are decanted and tamped lightly with an absorbent substrate. Non-specific binding is then blocked. In order to achieve this, a block buffer, is added to the wells. For example, a block buffer containing bovine serum albumin (BSA) such as that sold by InterGen Company, Purchase, NY, is suitable. It has been found that addition of between about 100 to 200µl of block buffer to each well followed by gentle agitation at room temperature for between about 1-2 hours is sufficient to block non-specific binding. It is also possible to add the block buffer directly to the cell lysate obtained in the previous step rather than to the second assay plate.

Following this, the capture agent-coated plates are washed several times (usually between about 3-8 times) with a wash buffer. The wash buffer can comprise phosphate buffered saline (PBS) at pH 7.0 to 7.5, for example. However, other wash buffers are available which can also be used. Conveniently, an automated plate washer, such as the ScanWasher 300 (Skatron Instruments, Inc., Sterling, VA) can be used for this, and other, washing steps of the assay.

#### B. *Measuring tyrosine phosphorylation*

The activated, solubilized receptor construct is then added to the wells having the capture agent adhering thereto. As a general proposition, about 80% of cell lysate obtained as mentioned under Section above can be added to each well (*i.e.*, about 60 to 160 µl depending on the original volume of the wells). The lysate is incubated with the capture agent for an adequate period of time to enable the receptor construct to be captured in the wells, *e.g.*, from 1 to 3 hours. Incubation can be carried out at room temperature.

Unbound cell lysate is then removed by washing with wash buffer. Following this washing step, an amount of the anti-phosphotyrosine antibody which is equal to, or less than, the amount of block buffer added previously, is added to each well. For example, about 50 to 200µl of an anti-phosphotyrosine antibody preparation having between about 0.3 to 0.5 µg/ml of antibody in a suitable buffer (*e.g.*, PBS with a detergent such as those included in the lysis buffer) is added to the well. This is followed by a washing step to remove unbound anti-phosphotyrosine antibody.

Tyrosine phosphorylation is then quantified by the amount of anti-phosphotyrosine antibody binding to the second solid phase. Many systems for detecting the presence of an antibody are available to those skilled in the art. Some examples follow.

Generally, the anti-phosphotyrosine antibody will be labelled either directly or indirectly with a detectable label. Numerous labels are available which can be generally grouped into the following categories:

(a) Radioisotopes, such as  $^{35}\text{S}$ ,  $^{14}\text{C}$ ,  $^{125}\text{I}$ ,  $^3\text{H}$ , and  $^{131}\text{I}$ . The antibody can be labeled with the radioisotope using the techniques described in Current Protocols in Immunology, *supra*, for example and radioactivity can be measured using scintillation counting.

(b) Fluorescent labels such as rare earth chelates (europium chelates) or fluorescein and its derivatives, rhodamine and its derivatives, dansyl, Lissamine, phycoerythrin and Texas Red are available. The fluorescent labels can be conjugated to the antibody using the techniques disclosed in Current Protocols in Immunology, *supra*, for example. Fluorescence can be quantified using a fluorimeter (Dynatech).

(c) Various enzyme-substrate labels are available and U.S. Patent No. 4,275,149 provides a review of some of these. The enzyme generally catalyses a chemical alteration of the chromogenic substrate which can be measured using various techniques. For example, the enzyme may catalyze a color change in a substrate, which can be measured spectrophotometrically. Alternatively, the enzyme may alter the fluorescence or chemiluminescence of the substrate. Techniques for quantifying a change in fluorescence are described above. The chemiluminescent substrate becomes electronically excited by a chemical reaction and may then emit light which can be measured (using a Dynatech ML3000 chemiluminometer, for example) or donates energy to a fluorescent acceptor. Examples of enzymatic labels include luciferases (*e.g.*, firefly luciferase and bacterial luciferase; U.S. Patent No. 4,737,456), luciferin, 2,3-dihydrophthalazinediones, malate dehydrogenase, urease, peroxidase such as horseradish peroxidase (HRPO), alkaline phosphatase,  $\beta$ -galactosidase, glucoamylase, lysozyme, saccharide oxidases (*e.g.*, glucose oxidase, galactose oxidase, and glucose-6-phosphate dehydrogenase), heterocyclic oxidases (such as uricase and xanthine oxidase), lactoperoxidase, microperoxidase, and the like. Techniques for conjugating enzymes to antibodies are described in O'Sullivan *et al.*, Methods for the Preparation of Enzyme-Antibody Conjugates for use in Enzyme Immunoassay, in Methods in Enzym. (ed J. Langone & H. Van Vunakis), Academic press, New York, 73: 147-166 (1981) and Current Protocols in Immunology, *supra*.

Examples of enzyme-substrate combinations include, for example:

(i) Horseradish peroxidase (HRPO) with hydrogen peroxide as a substrate, wherein the hydrogen peroxidase oxidizes a dye precursor (*e.g.* orthophenylene diamine (OPD) or 3,3',5,5'-tetramethyl benzidine hydrochloride (TMB)).

(ii) alkaline phosphatase (AP) with para-Nitrophenyl phosphate as chromogenic substrate.

(iii)  $\beta$ -D-galactosidase ( $\beta$ -D-Gal) with a chromogenic substrate (*e.g.* p-nitrophenyl- $\beta$ -D-galactosidase) or fluorogenic substrate 4-methylumbelliferyl- $\beta$ -D-galactosidase.

Numerous other enzyme-substrate combinations are available to those skilled in the art. For a general review of these, see U.S. Patent Nos. 4,275,149 and 4,318,980.

Sometimes, the label is indirectly conjugated with the antibody. The skilled artisan will be aware of various techniques for achieving this. For example, the antibody can be conjugated with biotin and any of the three broad categories of labels mentioned above can be conjugated with avidin, or *vice versa*. Biotin binds selectively to avidin and thus, the label can be conjugated with the antibody in this indirect manner. See, Current Protocols in Immunology, *supra*, for a review of techniques involving biotin-avidin conjugation. Alternatively, to achieve indirect conjugation of the label with the antibody, the antibody is conjugated with

a small hapten (*e.g.* digoxin) and one of the different types of labels mentioned above is conjugated with an anti-hapten antibody (*e.g.* anti-digoxin antibody). Thus, indirect conjugation of the label with the antibody can be achieved.

In another embodiment of the invention, the anti-phosphotyrosine antibody need not be labeled, and the presence thereof can be detected using a labeled anti-antiphosphotyrosine antibody (*e.g.* anti-mouse anti-phosphotyrosine antibody conjugated with HRPO).

In the preferred embodiment, the anti-phosphotyrosine antibody is labeled with an enzymatic label which catalyzes a color change of a substrate (such as tetramethyl benzimidine (TMB), or orthaphenylene diamine (OPD)). Thus, the use of radioactive materials is avoided. A color change of the reagent can be determined spectrophotometrically at a suitable wavelength (*e.g.* 450nm for TMB and 490nm for OPD, with a reference wavelength of 650 nm).

### 3. Intracellular Kinase Activity

The assay described herein is also used by measuring phosphorylation and/or activation of an intracellular kinase domain (*e.g.* form a cytoplasmic tyrosine kinases and/or cytoplasmic serine-threonine kinases) fused to the  $\alpha$ -subunit receptor. Phosphorylation of these molecules can occur as a consequence of trans-phosphorylation of the intracellular kinase domain by a kinase receptor or "receptor complex" (which comprises one or more kinase receptors residing in a cell membrane). Examples of intracellular tyrosine kinases include insulin receptor substrate I (IRS-1), Shc, Ras and GRB2, for example. Antibodies to human Shc, human Ras and GRB2 can be obtained commercially from UBI, NY, which can be used as capture agents for these tyrosine kinases. Examples of intracellular serine-threonine kinases include MEK and MAPK.

In order to measure phosphorylation of receptor constructs containing catalytic domains from these kinases, the procedure is essentially as described above, the chimera referred to as a "kinase construct." Generally, a eukaryotic cell will be transformed with nucleic acid encoding a kinase construct. Upon expression of the nucleic acid, the kinase construct will reside intracellularly (*i.e.* in the cytoplasm). The cells comprising the kinase construct are subjected to the KIRA as discussed above. Exposure to the agonist may result in trans-phosphorylation of the intracellular kinase construct which can be quantified in the ELISA as elaborated above. The capture agent in the ELISA binds to either the intracellular kinase construct or to the flag polypeptide.

### 4. Serine-Threonine Kinase Activity

This assay is further used by measuring for phosphorylation and/or activation of serine-threonine kinase ICD domain fused to the  $\alpha$ -subunit receptor. The term "serine-threonine kinase" refers to a kinase which phosphorylates a substrate which has at least one phosphate accepting alcohol group. The serine-threonine kinase is usually a "receptor" insofar as it has a ligand-binding ECD, TM domain and ICD. The ICD usually comprises a catalytic kinase domain and generally has one or more phosphate accepting serine and/or threonine residues. Examples of intracellular serine-threonine kinases include MEK and MAPK. Measuring phosphorylation of intracellular serine-threonine kinases can be done as described herein. Examples of serine-threonine kinase receptors that can provide suitable ICD domains for fusion to create a

receptor construct include *daf-1*, activin type II receptor (ActR-II), activin type IIB receptor (ActR-IIB), TGF- $\beta$  type II receptor (T $\beta$ R-II), activin receptor-like kinase (ALK) -1, -2, -3, -4 and TGF- $\beta$  type I receptor (T $\beta$ R-1)/ALK-5. See ten Dijke *et al.*, *supra*. The serine-threonine kinase assay is essentially the same as described above for tyrosine kinases, except that phosphorylation is quantified using anti-phosphoserine and/or anti-phosphothreonine antibodies. Anti-phosphoserine and anti-phosphothreonine monoclonal antibodies can be purchased from Sigma Immuno Chemicals, St Louis, MO, for example.

#### 5. Phosphatase Activity

Phosphatase activity can similarly be measured using the assay described herein. Phosphatase enzymes are able to dephosphorylate phosphorylated tyrosine, serine and/or threonine residues (*i.e.* liberate inorganic phosphate from phosphoric esters of such amino acid residues). Generally the phosphatase enzyme is specific for either tyrosine residues or serine-threonine residues but sometimes can dephosphorylate tyrosine, serine and threonine residues. Sometimes "endogenous" phosphatase activity is measured and this refers to the activity of phosphatase enzyme(s) which exist in nature in a selected cell. In order to quantify endogenous phosphatase activity, cells possessing at least one phosphatase are stimulated in the presence and absence of one or more phosphatase inhibitors. Examples of protein tyrosine phosphatase (PTPase) inhibitors include sodium orthovanadate and sodium molybdate (Sigma Chemical Co., St. Louis, MO). ICN Biochemicals supply okadaic acid which is a serine-threonine phosphatase inhibitor. As a general proposition, between about 1-10 $\mu$ M phosphatase inhibitor can be added to each well of the assay plate. In all other respects, the assay is performed essentially as discussed above. Thus, the ability of endogenous phosphatases to dephosphorylate a kinase in the selected cell can be quantified.

In the preferred embodiment, a phosphatase enzyme of interest can be studied. Examples of protein tyrosine phosphatases (PTPases) include PTP1B, PTPMEG, PTP1c, Yop51, VH1, cdc25, CD45, HLAR, PTP18, HPTP $\alpha$  and DPTP10D. See Zhang and Dixon, Adv. Enzym. 68: 1-36 (1994). Examples of protein serine-threonine phosphatases include PP1, PP2A, PP2B and PP2C. See Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:389-398 (1991). These proteins can be purchased commercially or made using the recombinant techniques described herein. To measure phosphatase activity, the KIRA ELISA can be performed essentially as described above with the following modifications. Following capture of the kinase or kinase construct (*e.g.* receptor construct) to the second solid phase and the washing step (to remove unbound cell lysate), the phosphatase of interest is added to the wells of the second assay plate and incubated with the adhering kinase or kinase construct. For example, between about 50-200  $\mu$ l of the phosphatase in a suitable dilution buffer (see Meth. Enzym., ed Hunter & Sefton, Academic press, New York, 201:416-440 (1991)) can be added to each well. This is generally followed by gentle agitation at room temperature (or 37°C) for between about 30 min to 2 hours to allow the phosphatase to dephosphorylate the kinase. Following washing to remove the phosphatase, the decreased degree of phosphorylation of the kinase relative to the control (*i.e.* no phosphatase added) is quantified by ELISA as described earlier herein.

#### 6. Kits

As a matter of convenience, the reagents can be provided in a kit, *i.e.*, a packaged combination of reagents, for combination with the analyte in assaying the ability of the analyte to activate or

prevent activation of a  $\alpha$ -subunit receptor of interest. The components of the kit will be provided in predetermined ratios. Thus, a kit will comprise the specific second solid phase for the assay as well as the anti-flag polypeptide capture agent either packaged separately or captured to the second solid phase (*e.g.* a microtiter plate). Usually, other reagents, such as the anti-phosphotyrosine antibody labelled directly or indirectly with an enzymatic label will also be provided in the kit. Where the detectable label is an enzyme, the kit will include substrates and cofactors required by the enzyme (*e.g.* a substrate precursor which provides the detectable chromophore or fluorophore). In addition, other additives may be included such as stabilizers, buffers (*e.g.* a block buffer and a lysis buffer) and the like. Conveniently, the kit can also supply the homogeneous population of cells which have been transformed with the receptor construct. The relative amounts of the various reagents may be varied widely to provide for concentrations in solution of the reagents which substantially optimize the sensitivity of the assay. Particularly, the reagents may be provided as dry powders, usually lyophilized, including excipients which on dissolution will provide a reagent solution having the appropriate concentration. The kit also suitably includes instructions for carrying out the KIRA ELISA.

#### 7. Uses for the Assay

This application provides two assays which are useful for reliable, sensitive and quantitative detection of kinase activation, which reflects ligand-binding by an  $\alpha$ -subunit receptor, caused by its homo-dimerization or homo-oligomerization. The first assay can be used where a receptor-specific capture antibody having the desired characteristics herein described is available or has been prepared. The second assay is a generic assay which enables activation of any receptor construct to be measured via the use of a flag polypeptide and a capture agent which binds specificity thereto.

These assays are useful for identifying novel agonists/antagonists for a selected receptor. Also, the assay provides a means for studying ligand-receptor interactions (*i.e.*, mechanism studies). Also the presence of an endogenous receptor in a selected cell line can be quantified using the assay. The assays are further useful for identifying the presence of a ligand for a selected receptor in a biological sample and, *e.g.*, establishing whether a growth factor has been isolated following a purification procedure. It is desirable to have an assay for measuring the ability of these growth factors to activate their respective receptors.

The assay also has clinical applications for detecting the presence of a ligand for a selected receptor (*e.g.* the GFR $\alpha$ 3 receptor) in a biological sample taken from a human and thus patients having elevated or depressed levels of the ligand can be identified. This is particularly desirable where elevated or depressed levels of the ligand cause a pathological condition. Accordingly, candidates for administration of the selected ligand (*e.g.* insulin) can be identified through this diagnostic method. It is possible, using the assay disclosed herein, to assay the pK of agonists or antagonists administered to a patient. This assay also facilitates the detection of shed receptor in a biological sample.

The assay is also useful to quantify phosphatase activity of endogenous phosphatases or, in the preferred embodiment, a phosphatase of interest. This can be used for screening phosphatase inhibitors, for example.



The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

### EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

#### Example 1

##### 10 Cloning of Mouse GFR $\alpha$ 3

Using sequences from the neurturin receptor, now known as GFR $\alpha$ 2 ("glial-cell-line-derived neurotrophic factor family receptor alpha"), a novel, potential member of the GFR $\alpha$  family was identified as a mouse EST in a public gene database (Accession Numbers W99197 (SEQ ID NO: 1), AA041935 (SEQ ID NO: 2) and AA050083 (SEQ ID NO: 3)). A DNA fragment corresponding to this potentially new receptor was obtained by Marathon PCR using mouse E15 cDNA (Clontech, Inc. USA) as template and PCR primers derived from the mouse EST. The PCR product was then used to screen a lambda gt10 mouse E15 library (Clontech, Inc. USA) to obtain a full length clone. The nucleotide sequence of the full length mouse cDNA is provided as SEQ ID NO: 4 (Figure 1A-1B). The protein sequence (SEQ ID NO: 5; see Figure 1A-1B) encoded by the isolated DNA was designated GFR $\alpha$ 3, since it was determined to be a novel protein with sequence identity to GFR $\alpha$ 1 (formerly the GDNF Receptor alpha) and GFR $\alpha$ 2 (formerly the Neurturin Receptor alpha; NTN $\alpha$ ). A comparison of the 397 amino acid mouse GFR $\alpha$ 3 protein sequence (SEQ ID NO: 5) to rat GFR $\alpha$ 1 (SEQ ID NO: 8) and rat GFR $\alpha$ 2 (SEQ ID NO: 9) is provided in Figure 2. The mGFR $\alpha$ 3 sequence is believed to identify a novel series of homologs belonging to the GFR receptor family. Potential N-linked glycosylation sites are shown shaded in Figure 2. The hydrophobic sequence involved in GPI-anchoring is overlined in Figure 2, with possible GPI attachment site indicated by the asterisks. A variant of the mouse GFR $\alpha$ 3 DNA contains a deletion of the "T" base at position 290 in Figure 1A, resulting in a frameshift and truncation protein variant. The variant DNA is nonetheless useful for hybridization, diagnostics, and other uses of the DNA (excluding full-length protein production) discussed throughout this specification. DNA (positions 89-289) comprising the GFR $\alpha$ 3 coding region immediately upstream of this base find use in the invention. DNA comprising the sequence immediately downstream (291-1279) provide another useful embodiment of the invention.

*In situ* hybridization studies using DNA encoding mouse GFR $\alpha$ 3 revealed a pattern of expression in peripheral sensory neurons and sympathetic neurons (data not shown).

## Example 2

### Isolation of cDNA Clones Encoding Human GFR $\alpha$ 3

To identify rapidly whether a human homolog of this new receptor might exist, an expressed sequence tag (EST) DNA database (a proprietary EST database, LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) was searched and an EST (INC3574209) was identified having the sequence:

GCGCTGNNTGNCNGNANGNGGGGGCGGGAGGTGCCGGTCGAGGGAGCCCCGCTCTCAGAG  
CTCCAGGGGAGGAGCGANGGGAGCGCGGAGCCCGGCCCTACAGCTCGCCATGGTGCGC  
CCCCTGAACCCGCGACCGCTGCCGCCGTAGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN  
NNNNNNNGCCTCTCGCAGCCGGAGACCCCTTCCCACAGAAAGCCGACTCATGAACAGC  
TGTCTCCAGGCCAGGAGGAAGTGCCAGGCTGATCCCACCTGC (SEQ ID NO: 10).

This sequence had 61% identity to the murine GFR $\alpha$ 3.

To clone the corresponding full length cDNA, a panel of cDNA libraries were screened with primers:

newa3.F            5' GCC TCT CGC AGC CGG AGA CC 3' (SEQ ID NO: 11)

newa3.R            5' CAG GTG GGA TCA GCC TGG CAC 3' (SEQ ID NO: 12)

DNA from the libraries was screened by PCR amplification, as per *Ausubel et al., Current Protocols in Molecular Biology (1995)*, with the PCR primer pair. A strong PCR product was identified in all libraries analyzed (fetal lung, fetal kidney, and placenta).

To isolate a cDNA clone encoding this protein, a human fetal lung-pRK5 vector library was selected and enriched for positive cDNA clones by extension of single stranded DNA from plasmid libraries grown in dug-/bung- host using the newa3.R primer. RNA for construction of the cDNA libraries was isolated from human fetal lung tissue. The cDNA library used to isolate the cDNA clones was constructed by standard methods using commercially available reagents (e.g., Invitrogen, San Diego, CA; Clontech, etc.). The cDNA was primed with oligo dT containing a NotI site, linked with blunt to SalI hemikinased adaptors, cleaved with NotI, sized appropriately by gel electrophoresis, and cloned in a defined orientation into a suitable cloning vector (pRKB or pRKD; pRK5B is a precursor of pRK5D that does not contain the SfiI site; see, Holmes et al., *Science*, 253:1278-1280 (1991)) in the unique XhoI and NotI sites. To enrich for positive cDNA clones the primer extension reaction contained 10 $\mu$ l of 10x PCR Buffer (Perkin Elmer, USA), 1 $\mu$ l dNTP (20mM), 1 $\mu$ l library DNA (200 ng), 1  $\mu$ l primer, 86.5 $\mu$ l H<sub>2</sub>O and 1 $\mu$ l of Amplitaq (Perkin Elmer, USA) added after a hot start. The reaction was denatured for 1 minute at 95°C, annealed for 1 minute at 60°C, and then extended for 15 minutes at 72°C. The DNA was extracted with phenol/chloroform, precipitated with ethanol, and then transformed by electroporation into a DH10B host bacteria. The entire transformation mixture was plated onto 10 plates and colonies were allowed to form. Colonies were lifted onto nylon membranes and screened with an oligonucleotide probe (newa3.probe: 5' TCT CGC AGC CGG AGA CCC CCT TCC CAC AGA AAG CCG ACT CA 3' (SEQ ID NO: 13)) derived from the Incyte EST. Filters were hybridized with the probe overnight at 42°C in 50% formamide, 5xSSC, 10xDenhardt's, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, and 50  $\mu$ g/ml of sonicated salmon sperm DNA. Filters were then rinsed in 2xSSC,

washed in 0.1xSSC, 0.1% SDS, and then exposed overnight to Kodak X Ray films. Five positive clones were identified. Pure positive clones were obtained after colony purification and secondary screening.

Two of the isolated clones were sequenced. These cDNA sequences were designated DNA48613 (SEQ ID NO: 14) and DNA48614 (SEQ ID NO: 16). Amino acid sequence analysis of DNA48613 revealed a 400 amino acid long open reading frame sequence (SEQ ID NO: 15) with a predicted 26 amino acid long N-terminal signal peptide. The predicted mature protein is 374 amino acids long, with a calculated molecular weight of approximately 41 kDa. Potential N-linked glycosylation sites are similar to those in the mouse sequence. The mouse and human GFR $\alpha$ 3 protein sequences are compared in Figure 3.

The deduced amino acid sequence (SEQ ID NO: 17) of DNA48614 and comparison to SEQ ID NO: 15, revealed it to be an alternatively spliced form of DNA48613, with a 30 amino acid deletion (amino acid positions 127-157, counting from the initiation methionine), as shown in Figure 4.

Interestingly, none of the cysteines are deleted in this clone. Clones DNA48613, DNA48614 and mGFR $\alpha$ 3(clone 13) variant have been deposited with ATCC and are assigned ATCC deposit nos. 209752 (Designation: DNA48613-1268), 209751 (Designation: DNA48614-1268), and \_\_\_\_\_, respectively.

A comparison of the nucleic acid sequences encoding DNA48613 with those encoding human GFR $\alpha$ 1 and GFR $\alpha$ 2 is provided in Figures 5A-B. The 5' untranslated GFR $\alpha$ 3 sequence immediately upstream of the initiation ATG in the cloned DNA48613 is

GCGAGGGGAGCGCGGAGCCCGGCGCCTACAGCTCGCC (SEQ ID NO 21).

As discussed below, a sequence comparison of the protein encoded by DNA48613 to GFR $\alpha$ 1 and GFR $\alpha$ 2 (Figure 6) indicated that the two human proteins are new members of the GFR $\alpha$  receptor family, and are human homologs of murine GFR $\alpha$ 3. Accordingly, DNA48613 encodes a protein designated human GFR $\alpha$ 3, and DNA48614 encodes its splice variant.

Amino acid sequence comparisons between GFR $\alpha$  family members are provided in Table 1, based on a BLAST-2 and FastA sequence alignment analysis of the full-length sequence.

**Table 1**

**Sequence Identity Between Members of the GFR $\alpha$  Family**

<u>Proteins Compared</u>	<u>Percent Identity</u>
rGFR $\alpha$ 1 versus hGFR $\alpha$ 1	92%
rGFR $\alpha$ 2 versus hGFR $\alpha$ 2	94%
mGFR $\alpha$ 3 versus hGFR $\alpha$ 3	77%
hGFR $\alpha$ 3 versus hGFR $\alpha$ 1	34%
hGFR $\alpha$ 3 versus hGFR $\alpha$ 2	34%
hGFR $\alpha$ 1 versus hGFR $\alpha$ 2	48%

From the sequence comparisons it can be seen that human GFR $\alpha$ 3 is less related to its rodent homologue than is either GFR $\alpha$ 1 or GFR $\alpha$ 2. In addition, GFR $\alpha$ 3 appears to be more distantly related to GFR $\alpha$ 1 and GFR $\alpha$ 2 than GFR $\alpha$ 1 and GFR $\alpha$ 2 are to each other.

**Example 3**

**Use of GFR $\alpha$ 3 as a hybridization probe**

The following method describes use of a nucleotide sequence encoding GFR $\alpha$ 3 as a hybridization probe.

DNA comprising the coding sequence of GFR $\alpha$ 3 (shown in SEQ ID NO: 4, SEQ ID NO: 14 or SEQ ID NO: 16), or a fragment thereof, is employed as a probe to screen for homologous DNAs (such as those encoding naturally-occurring GFR $\alpha$ 3 or variants of GFR $\alpha$ 3) in human tissue cDNA libraries, human tissue genomic libraries, RNA isolated from tissues, tissue preparations in situ, or chromosome preparations (such as for chromosome mapping).

Hybridization and washing of filters containing either library DNAs is performed under the following high stringency conditions. Hybridization of radiolabeled GFR $\alpha$ 3 -derived probe to the filters is performed in a solution of 50% formamide, 5x SSC, 0.1% SDS, 0.1% sodium pyrophosphate, 50 mM sodium phosphate, pH 6.8, 2x Denhardt's solution, and 10% dextran sulfate at 42°C for 20 hours. Washing of the filters is performed in an aqueous solution of 0.1x SSC and 0.1% SDS at 42°C.

DNAs having a desired sequence identity with the DNA encoding full-length native sequence GFR $\alpha$ 3 can then be identified using standard techniques known in the art.

#### Example 4

##### Northern Blot Analysis

Expression of GFR $\alpha$ 3 mRNA in human tissues was examined by Northern blot analysis. Multiple human tissue RNA blots were hybridized to a <sup>32</sup>P-labelled DNA probe encompassing the entire coding region of human GFR $\alpha$ 3 cDNA labelled by random priming. Human fetal RNA blot MTN (Clontech, Inc. USA) and human adult RNA blots MTN-I and MTN-II (Clontech) were incubated with the DNA probe. Blots were incubated with 1x10<sup>6</sup> cpm/ml probe in hybridization buffer (5X SSC; 10X Denhardt's solution; 0.05M sodium phosphate pH 6.5, 50 µg/mL sonicated salmon sperm DNA; 50% formamide; 0.1% sodium pyrophosphate) for 42°C overnight. The blots were washed in 2X SSC at room temperature for 10 minutes followed by 0.2xSSC in 0.1% SDS at 42°C for 30 minutes. The blots were exposed to x-ray film and developed after overnight exposure by phosphorimager analysis (Fuji).

As shown in Fig.7 GFR $\alpha$ 3 mRNA transcripts were detected. Expression was observed at high levels in the heart, gut (pancreas, small intestine, colon), thymus, testis and prostate.

#### Example 5

##### Localization of GFR $\alpha$ 3 by In Situ Hybridization

The following tissues were surveyed for expression of GFR $\alpha$ 3 mRNA by *in situ* hybridization: day 13 mouse embryo, day 15 and day 17 embryonic mouse brain, postnatal day 1 mouse brain, adult mouse brain (with optic nerve), adult mouse spinal cord, adult mouse trigeminal ganglion and roots, adult mouse retina, and embryonic utricle of several stages.

For in-situ hybridization, E13.5 mouse embryos were immersion-fixed overnight at 4°C in 4% paraformaldehyde, cryoprotected overnight in 15% sucrose, embedded in O.T.C., and frozen on liquid nitrogen. Adult mouse spinal cord, trigeminal ganglia, retina, and P1 mouse brains were embedded in O.T.C. and fresh frozen on liquid nitrogen. Adult mouse brains were fresh frozen with powdered dry ice. Sections were cut at 16µm, and processed for in-situ hybridization for GFR $\alpha$ 3

by a method described previously (Phillips *et al.*, *Science* 250:290 (1990)). Using 33P-UTP, labeled RNA probes were generated as described (Melton *et al.*, *Nucleic Acids Res.* 12:7035 (1984)) using T7 polymerase with a 326 bp fragment encoding mouse GFR $\alpha$ 3.

In the E13 mouse, GFR $\alpha$ 3 mRNA was very strongly expressed in dorsal root ganglia, in sympathetic ganglia, and in peripheral nerves. The vestibular ganglion also displayed strong signal. Moderate expression was seen in whisker pads, in the region of the axilla, and surrounding the urinary bladder. Moderate expression was also seen in the intermediolateral region of the gray matter of the thoracic spinal cord, the ventromedial hypothalamus, and cell clusters in the dorsal hindbrain. Most other regions of the brain were devoid of demonstrable signal. Many other organs expressed either weak or undetectable signal, including lung, heart, liver, gut, and kidney.

At later developmental stages (E15, E17, P1, adult), GFR $\alpha$ 3 expression within the CNS was very limited. Most regions of brain and spinal cord demonstrated no hybridization signal above the background level seen in control sections hybridized with sense strand control probe. Exceptions to this were cell clusters found in the hindbrain. In the adult, a subpopulation of trigeminal ganglion neurons was very strongly labeled, while no labeling was seen in either satellite cells or in the nerve roots. The optic nerve also failed to demonstrate detectable signal.

In sections of adult mouse heart, there was diffuse signal over atrial and ventricular myocytes with focal areas of increased signal associated with the cardiac conduction system.

A comparison of labeling with GFR $\alpha$ 1, GFR $\alpha$ 2 and GFR $\alpha$ 3 is shown in Figure 8. The expression of GFR $\alpha$ 3 is very limited and localized in comparison to the other receptors.

Primers containing sense sequence GCCCGCGACCTCCACTGCTG (designated gfrp1; SEQ ID NO: 22) and antisense sequence CTGTGGGAGCGGCGGCG (designated gfrp2.r.c; SEQ ID NO: 23) were used to generate a 671 bp hybridization probe from the mouse GFR $\alpha$ 3. Primers containing sense sequence CCTGAACCTATGGTAACTGG (SEQ ID NO: 24) and antisense sequence ACCCAGTCCTCCCTACC (SEQ ID NO: 25) were used to generate a 378 bp hybridization probe from the mouse GFR $\alpha$ 3.

Human fetal tissues at E12-E16 weeks that were examined included placenta, umbilical cord, liver, kidney, adrenals, thyroid, lungs, heart, great vessels, esophagus, stomach, small intestine, spleen, thymus, pancreas, brain, spinal cord, body wall, pelvis and lower limb. Adult tissues examined included kidney (normal and endstage), adrenal, myocardium, aorta, lung, skin, eye (including retina), bladder, liver (normal, cirrhotic, and acute failure), renal carcinoma, and soft tissue sarcoma. Non-human primate tissues examined included chimpanzee salivary gland, stomach, thyroid, parathyroid, skin and thymus. Hybridization to the 378 base pair antisense strand probe was detected in fetal and adult human DRG's, peripheral nerves (as seen in the body wall and lower limb of the fetus) and mesenteric nerves in the fetus. No expression was observed in the fetal spinal cord or brain. No expression was observed in the neuroblastomas examined.

Using the 671 base pair antisense probe, GFR $\alpha$ 3 mRNA hybridization was detected in the early and late and adult rat in E14 ganglion, trigeminal, peripheral nerves of skin and skeletal muscle: E17 skin, dorsal root ganglion, peripheral nerves, cartilage, skeletal muscle, and brain; E19 dorsal root ganglion, peripheral nerves, brain, stratum corneum of skin, tooth, skeletal muscle, cartilage, liver and gut. No specific signal was

detected in fetal or adult rat pancreas. In all the examples in this section, the corresponding sense probes failed to hybridize as might be expected.

#### Example 6

##### Expression of GFR $\alpha$ 3 in *E. coli*

5       The DNA sequence encoding GFR $\alpha$ 3, e.g. human GFR $\alpha$ 3, is initially amplified using selected PCR primers. The primers should contain restriction enzyme sites that correspond to the restriction enzyme sites on the selected expression vector. A variety of expression vectors may be employed. An example of a suitable vector is pBR322 (derived from *E. coli*; see Bolivar *et al.*, *Gene*, 2:95 (1977)) which contains genes for ampicillin and tetracycline resistance. The vector is digested with restriction enzyme and  
10       dephosphorylated. The PCR amplified sequences are then ligated into the vector. The vector will preferably include sequences that encode for an antibiotic resistance gene, a trp promoter, a polyhis leader (including the first six STII codons, polyhis sequence, and enterokinase cleavage site), the mammalian GFR $\alpha$ 3 coding region, lambda transcriptional terminator, and an argU gene.

      The ligation mixture is then used to transform a selected *E. coli* strain using the methods described in  
15       Sambrook *et al.*, *supra*. Transformants are identified by their ability to grow on LB plates and antibiotic resistant colonies are then selected. Plasmid DNA can be isolated and confirmed by restriction analysis and DNA sequencing.

      Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a larger scale culture. The cells are  
20       then grown to a desired optical density, during which the expression promoter is turned on.

      After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized mammalian GFR $\alpha$ 3 protein can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

#### 25       Example 7

##### Expression of GFR $\alpha$ 3 in mammalian cells

      This example illustrates preparation of a glycosylated form of mammalian GFR $\alpha$ 3 by recombinant expression in mammalian cells.

      The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector.  
30       Optionally, the GFR $\alpha$ 3 DNA is ligated into pRK5 with selected restriction enzymes to allow insertion of the GFR $\alpha$ 3 DNA using ligation methods such as described in Sambrook *et al.*, *supra*. The resulting vector is called pRK5-GFR $\alpha$ 3.

      In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum  
35       and optionally, nutrient components and/or antibiotics. About 10  $\mu$ g pRK5-GFR $\alpha$ 3 DNA is mixed with about 1  $\mu$ g DNA encoding the VA RNA gene (Thimmappaya *et al.*, *Cell*, 31:543 (1982)) and dissolved in 500  $\mu$ l of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M CaCl<sub>2</sub>. To this mixture is added, dropwise, 500  $\mu$ l of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM NaPO<sub>4</sub>, and a precipitate is allowed to form for 10 minutes at 25°C.

The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200  $\mu\text{Ci/ml}$   $^{35}\text{S}$ -cysteine and 200  $\mu\text{Ci/ml}$   $^{35}\text{S}$ -methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of mammalian *GFR $\alpha$ 3* polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, mammalian *GFR $\alpha$ 3* may be introduced into 293 cells transiently using the dextran sulfate method described by Sompanyrac *et al.*, *Proc. Natl. Acad. Sci.*, 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700  $\mu\text{g}$  pRK5-*GFR $\alpha$ 3* DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5  $\mu\text{g/ml}$  bovine insulin and 0.1  $\mu\text{g/ml}$  bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed mammalian *GFR $\alpha$ 3* can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

In another embodiment, mammalian *GFR $\alpha$ 3* can be expressed in CHO cells. The pSUI-*GFR $\alpha$ 3* can be transfected into CHO cells using known reagents such as  $\text{CaPO}_4$  or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as  $^{35}\text{S}$ -methionine. After determining the presence of mammalian *GFR $\alpha$ 3* polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed mammalian *GFR $\alpha$ 3* can then be concentrated and purified by any selected method.

Epitope-tagged mammalian *GFR $\alpha$ 3* may also be expressed in host CHO cells. The mammalian *GFR $\alpha$ 3* may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into an expression vector. The poly-his tagged mammalian *GFR $\alpha$ 3* insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged mammalian *GFR $\alpha$ 3* can then be concentrated and purified by any selected method, such as by  $\text{Ni}^{2+}$ -chelate affinity chromatography.

#### Example 8

##### Expression of *GFR $\alpha$ 3* in Baculovirus-Infected Insect Cells

The following method describes recombinant expression of *GFR $\alpha$ 3* in Baculovirus-infected insect cells.

The GFR $\alpha$ 3 was fused upstream of an epitope tag contained within a Baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). The amino acid sequence of the GFR $\alpha$ 3-IgG fusion is provided in SEQ ID NO: 18. A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, GFR $\alpha$ 3 sequence encoding the extracellular domain) was amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer incorporate flanking (selected) restriction enzyme sites. The product was then digested with those selected restriction enzymes and subcloned into the expression vector. The vector for expression of GFR $\alpha$ 3-IgG in insect cells was pb.PH (where expression in Baculovirus was under control of the polyhedrin promoter).

Recombinant Baculovirus was generated by co-transfecting the above plasmid and BaculoGold<sup>TM</sup> virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses were harvested and used for further amplifications. Viral infection and protein expression was performed as described by O'Reilley *et al.*, "Baculovirus expression vectors: A laboratory Manual," Oxford: Oxford University Press (1994). Purification of the IgG tagged (or Fc tagged) GFR $\alpha$ 3 was performed using known chromatography techniques, including Protein A or protein G column chromatography

Alternatively, expressed poly-his tagged GFR $\alpha$ 3 can be purified, for example, by Ni<sup>2+</sup>-chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert *et al.*, *Nature* 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM MgCl<sub>2</sub>; 0.1 mM EDTA; 10% Glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45  $\mu$ m filter. A Ni<sup>2+</sup>-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to baseline A<sub>280</sub> with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which elutes nonspecifically bound protein. After reaching A<sub>280</sub> baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or western blot with Ni<sup>2+</sup>-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His<sub>10</sub>-tagged GFR $\alpha$ 3 are pooled and dialyzed against loading buffer.

#### Example 9

##### Binding to GFR $\alpha$ 3

In order to determine if any of the known GDNF family members (ligands GDNF, Neurturin (NTN) or Persephin (PSN)) could bind to GFR $\alpha$ 3, each ligand was coated onto microtiter plates and incubated with either GFR $\alpha$ 1-IgG, GFR $\alpha$ 2-IgG, or GFR $\alpha$ 3-IgG (SEQ ID NO: 18) prepared as in Example 8. Binding of GFR $\alpha$ -IgG was then detected with a secondary antibody to its IgG portion. GDNF, NTN, and PSN were coated onto microtiter plates at 1  $\mu$ g/ml in 50 mM carbonate buffer, pH 9.6, overnight at 4°C. The plates were



then washed with PBS/0.05% Tween 20, then blocked with PBS/0.05% BSA/0.05% Tween 20 for 1-2 hours at room temperature. Various concentrations of IgG-tagged chimeric receptors (GFR $\alpha$ 1-IgG, GFR $\alpha$ 2-IgG, GFR $\alpha$ 3-IgG; 1  $\mu$ g/ml to 1.95 ng/ml) were added to each well and the plates were incubated for 1 hour at room temperature. The plates were then washed as described above and incubated in the presence of goat anti-human IgG(Fc)-HRP (1:1000) for 1 hour at room temperature. After washing, bound HRP was revealed with OPD substrate for 5 to 10 minutes, followed by reading the plates at 490 nm. The results are shown in Figures 9A-C.

GFR $\alpha$ 1 binds to GDNF (Figure 9A), GFR $\alpha$ 2 binds to GDNF and NTN (Figure 9B), but GFR $\alpha$ 3 does not bind any of these molecules (Figure 9C). GFR $\alpha$ 3 is thus an orphan receptor.

#### Example 10

##### Assays for GFR $\alpha$ 3 agonists

The GDNF family of ligands uses a unique receptor system: a GPI-linked ligand binding protein ( $\alpha$ -component) and a signaling component, the tyrosine kinase receptor Ret. The mechanism of activation of this multicomponent receptor complex is still unknown, but tyrosine kinase receptors are known to be activated upon ligand-induced dimerization. Accordingly, a possible mechanism of GFR activation is by ligand binding to the  $\alpha$ -component which induces  $\alpha$ -component dimerization. The two  $\alpha$ -chains in turn will bring two Ret molecules into the complex, which will lead to activation of the kinase domains and phosphorylation of target tyrosine residues on the receptor and/or on other signaling molecules.

To demonstrate that ligands do induce dimerization of the  $\alpha$ -component, chimeric receptors made of the extracellular domain of the rat GFR $\alpha$ 2 and the transmembrane and intracellular domain of the TPO receptor (c-mpl) or of the Rse tyrosine kinase receptor were constructed. These two receptors belong to different family of receptors but are both known to be activated by ligand induced dimerization or by agonist-antibody-induced dimerization.

GFR $\alpha$ 2-c-mpl. A chimeric receptor made of the gD epitope tag followed by the rGFR $\alpha$ 2 extracellular domain (less the GPI signal) followed by the transmembrane and intracellular domain of the TPO receptor was assembled by recombinant PCR into the pRKtkneo vector under the control of the CMV promoter. Ba/F3 cells were electroporated with NotI linearized pRKtkneo-GFR $\alpha$ 2-mpl, and clones were obtained by limiting dilutions. Individual clones were analyzed for expression of the receptor by FACS analysis using an anti-gD antibody. Positive clones were selected and further characterized for their capacity to proliferate in response to NTN stimulation, a ligand for GFR $\alpha$ 2. As shown in Figure 10, Ba/F3 cells expressing GFR $\alpha$ 2-mpl are capable of proliferating in response to NTN stimulation, as assessed by 3H-thymidine incorporation.

GFR $\alpha$ 2-Rse. A chimeric receptor was constructed with the gD epitope tag followed by the rat GFR $\alpha$ 2 extracellular domain (less the GPI signal) followed by the transmembrane and intracellular domain of the Rse tyrosine kinase receptor and another gD epitope tag and was assembled by recombinant PCR into a pSVi vector under the control of the SV40 promoter. The gD-GFR $\alpha$ 2-Rse-gD sequence is presented in SEQ ID NO: 19. CHO cells were transfected by the lipofectamine method (GIBCO-BRL). Single transfected CHO clones were picked and analyzed for expression of the receptor by FACS analysis using an anti-gD antibody. Receptor-positive clones were then analyzed using a KIRA assay (e.g., U.S. Patent 5,709,858) for receptor

induced phosphorylation upon NTN stimulation. As shown in Figure 11, NTN stimulation caused autophosphorylation of the receptor.

**GFR $\alpha$ 3-Rse.** Together, the above data indicate that activation of the GFRs is mediated by ligand-induced dimerization and that, in addition to their ligands, the various receptors will be susceptible to antibody-mediated activation. Accordingly, in one embodiment, an assay to identify agonist antibodies and a natural ligand (or other agonists) for mammalian GFR $\alpha$ 3 follows the method described above for GFR $\alpha$ 2-Rse. A chimeric GFR $\alpha$ 3 receptor was constructed with the gD epitope tag followed by the murine GFR $\alpha$ 3 extracellular domain (less the GPI signal; preferably the human GFR $\alpha$ 3 is used) followed by the transmembrane and intracellular domain of the Rse tyrosine kinase receptor and a second gD tag using recombinant PCR into the pSVi vector under the control of the SV40 promoter. CHO cells were transfected by the lipofectamine method (GIBCO-BRL). Single transfected clones were picked and analyzed for expression of the GFR $\alpha$ 3 chimeric receptor by FACS analysis using an anti-gD antibody. Positive clones were then analyzed for receptor induced phosphorylation upon treatment with either GDNF, NTN or PSN. The results are shown in Figure 12. The results confirmed that GFR $\alpha$ 3 is a receptor for a novel ligand of the GDNF family. The sequence of gD-GFR $\alpha$ 3-Rse-gD is presented in SEQ ID NO: 20. As is evident from this construct sequence and its homology to the other GFR family members, a sufficient ligand binding region is from amino acid 110 to amino acid 386 of SEQ ID NO: 20, which corresponds to amino acid residues 84 to 360 in SEQ ID NO: 15. The natural ligand for GFR $\alpha$ 3 has been identified as artemin (Baloh et al., Neuron 21:1291-1302 (1998), which has been found to bind the GFR $\alpha$ 3 of the present invention and its gD-GFR $\alpha$ 3-Rse-gD fusion. Antibodies generated against GFR $\alpha$ 3 (or other candidate agonists) can be screened for agonist activity using the GFR $\alpha$ 3 construct expressed in CHO cells. Alternatively, antagonists are screened by their ability to inhibit agonist function in this assay.

### Example 11

#### Preparation of Antibodies that Bind GFR $\alpha$ 3

This example illustrates preparation of monoclonal antibodies which can specifically bind GFR $\alpha$ 3. Techniques for producing the monoclonal antibodies are known in the art and are described, for instance, in Goding, *supra*. Immunogens that may be employed include purified GFR $\alpha$ 3, fusion proteins containing GFR $\alpha$ 3, and cells expressing recombinant GFR $\alpha$ 3 on the cell surface. Selection of the immunogen can be made by the skilled artisan without undue experimentation. Mice, such as Balb/c, are immunized with the GFR $\alpha$ 3 immunogen emulsified in complete Freund's adjuvant and injected subcutaneously or intraperitoneally in an amount from 1-100 micrograms. Alternatively, the immunogen is emulsified in MPL-TDM adjuvant (Ribi Immunochemical Research, Hamilton, MT) and injected into the animal's hind foot pads. The immunized mice are then boosted 10 to 12 days later with additional immunogen emulsified in the selected adjuvant. Thereafter, for several weeks, the mice may also be boosted with additional immunization injections. Serum samples may be periodically obtained from the mice by retro-orbital bleeding for testing in ELISA assays to detect GFR $\alpha$ 3 antibodies.

After a suitable antibody titer has been detected, the animals "positive" for antibodies can be injected with a final intravenous injection of GFR $\alpha$ 3. Three to four days later, the mice are sacrificed and the spleen

cells are harvested. The spleen cells are then fused (using 35% polyethylene glycol) to a selected murine myeloma cell line such as P3X63AgU.1, available from ATCC, No. CRL 1597. The fusions generate hybridoma cells which can then be plated in 96 well tissue culture plates containing HAT (hypoxanthine, aminopterin, and thymidine) medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells will be screened in an ELISA for reactivity against GFR $\alpha$ 3. Determination of "positive" hybridoma cells secreting the desired monoclonal antibodies against GFR $\alpha$ 3 is within the skill in the art. The positive hybridoma cells can be injected intraperitoneally into syngeneic Balb/c mice to produce ascites containing the anti-GFR $\alpha$ 3 monoclonal antibodies. Alternatively, the hybridoma cells can be grown in tissue culture flasks or roller bottles. Purification of the monoclonal antibodies produced in the ascites can be accomplished using ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can be employed.

### Example 12

#### Dimerization Screening Assays

Candidate agonists, for example antibodies generated against the  $\alpha$ -subunit receptor of the GFR family, e.g. GFR $\alpha$ 3, GFR $\alpha$ 2, or GFR $\alpha$ 1, can be screened for agonist activity using the appropriate  $\alpha$ -receptor-Rse-like construct expressed in CHO cells or other suitable cells in a KIRA assay. An exemplary KIRA protocol using monoclonal antibodies to the gD portion of the gD-GFR $\alpha$ 2-Rse construct is presented.

CHO cell culture expressing the gD-GFR $\alpha$ 2-Rse fusion protein were prepared and cultured as follows. On Day 1 the transfected CHO cells from culture flask are preferably 70-90% confluent with very few floating (detached) cells. Culture plates were Falcon (1270) flat bottom, 96-well sterile tissue culture plates with cover. The Detachment Buffer was PBS with 1:50 diluted 5 mM EDTA (250 mM stock). The Cell Culture Media was Ham's F-12 without GHT, low Glucose DMEM without Glycine: with NaHCO<sub>3</sub> (50:50) + 10 % Diafiltered FBS, 25 mM HEPES, 2 mM L-Glutamine. On Day 2 the Stimulation Media was Excell-401 insect cell media (JRH Biosciences cat#14401-78p) plus 0.5 % BSA. The Lysis Buffer was 150mM NaCl with 50 mM HEPES and 0.5% Triton-X 100. Protease inhibitors added to lysis buffer before use were 100X AEBSF (100 mM) stock using 1:100 dilution, 1000X Aprotinin (liquid) stock using 1:1000 dilution, and 1000X Leupeptin (50 mM) stock using 1:1000 dilution. Phosphatase inhibitor added to lysis buffer before use was 50X Sodium Orthovanadate (100 mM) stock using 1:50 dilution.

The ELISA format used the following materials. Solid support was Nunc Maxisorp immunoplate 4-39454. Coating buffer was PBS pH 7.4. Washing Buffer was PBS with 0.05% tween 20 pH 7.4. Blocking Buffer was PBS with 0.5% BSA. Assay Buffer was PBS with 0.5% BSA, 0.05% Tween 20 and 5 mM EDTA, pH 7.4. Substrate was a TMB substrate kit (2 bottles: A: TMB substrate; B: TMB peroxide solution) from Kirkegard and Perry. Stopping Solution was 1.0 M H<sub>3</sub>PO<sub>4</sub>. Antigen was solublized transfected "Receptor.gD" from cell culture wells (cell lysate). Antibodies were (1°) 3C8 (anti-gD peptide) concentration 1.0  $\mu$ g/ml, 1:1300 dil 1.3 mg/ml stock, lot 24564-7 #1766, (2°) Biotinylated 4G10 (UBI) concentration 1 : 1000 from 4°C stock (50  $\mu$ g/ml) #100796. Conjugate was Streptavidin/HRP Zymed concentration 1 : 50000

lot #26246-91 (1:100 frozen stock ).

Cells were harvested by aspirating cell culture supernatant from tissue culture flask, rinsed once with sterile PBS, and 10 ml of the cell detachment buffer was added. The cells were incubated at 37°C for ~ 10 min until cells detached. Detached cells were transferred to a centrifuge tube and an equal volume of cell culture medium was added. Count cells were done with a hemocytometer. Cells were centrifuged, supernatant was removed by aspiration, and cells were suspended to 1x10<sup>6</sup> cells/ml. Added to each well was 100 µl cell suspension (final of about 1x10<sup>5</sup> cells / well). Plates were incubated at 37°C overnight.

Receptor activation was performed as follows. Typically, a stock of ligand, in this example a 2 mg/ml preparation of hNTNFP was used to make a final concentration of NTN in each well as 0.1 , 0.05, 0.025, 0.0125, 0.00625, 0.00312, 0.001, and 0.0 ug/ml. Solutions in microtiter plates were gently mixed by external shaking. A 100 µl of sample, control or NSB was added to each well and incubated for 25 minutes at 37°C. Gentle mixing of the plates was done. To each well was added 130 µl Lysis Buffer with protease and phosphatase inhibitors. Cell lysis was allowed to proceed for 30 minutes in the tissue culture plates. For storage the cell lysates were placed at -70°C.

An ELISA was run as follows. To coat the ELISA plates, 100 µl of 1° mAb (primary; 3C8 1µg/ml) solution was added to each well, and allowed to coat the wells at 4° C overnight. To perform the assay in Capture (ELISA) plates, the coat solution was discarded and 150 µl of blocking buffer was added to each well. Blocking was allowed to continue for 1 hour. Cell lysates were thawed with gentle agitation. The ELISA plates were rinsed with wash buffer three times (using a Skatron ScanWasher 300). To each capture ELISA plate well, 100 µl cell lysate was transferred using a fresh pipette tips for each transfer. Plates were incubated at room temperature for 2 hours with gentle agitation. Dilute biotinylated 4G10 (2° Ab; secondary antibody; 4°C) 1:1000 in assay buffer was prepared. Each well was rinsed 10 times with wash buffer. To each well was added 100 µl of 2° Ab, followed by incubation at room temperature for 2 hours with gentle agitation. Plates were washed with wash buffer six times. Dilute Streptavidin/HRP 1:50000 in assay buffer was prepared. To each well was added 100 µl diluted StrAv/HRP, followed by incubation at room temperature for 1 hour with gentle agitation. Plates were washed with wash buffer six times. To each well was added 100 µl of substrate solution: 1 volume of K&P TMB substrate plus 1 volume of K&P TMB peroxide solution. The reaction color was allowed to develop for 15 minutes, followed by an addition of 50 µl of 1.0 M H<sub>3</sub>PO<sub>4</sub> to quench the color development. The O.D. (450 nm) of each well was read. Figure 13 shows the activation results using three different agonist antibodies--in this case the antibodies were raised against the gD flag epitope, but were able to induce  $\alpha$ -subunit oligomerization and subsequent tyrosine kinase domain (Rse region) activation.

#### Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

<u>Material</u>	<u>ATCC Dep. No.</u>	<u>Deposit Date</u>
mGFR $\alpha$ 3(clone 13)	_____	_____
DNA48613	209752	April 07, 1998

DNA48614

209751

April 07, 1998

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR §1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

What is claimed is:

1. Isolated nucleic acid comprising a nucleic acid having at least a 65% sequence identity to (a) a nucleic acid molecule encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 27 to 400 of SEQ ID NO: 15 or the sequence of amino acids 27 to 369 of SEQ ID NO: 17, or (b) the complement of the nucleic acid molecule of (a).  
5
2. The isolated nucleic acid of claim 1 comprising a nucleic acid having at least a 65% sequence identity to (a) a nucleic acid molecule encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 1 to 400 of SEQ ID NO: 15 or the sequence of amino acids 1 to 379 of SEQ ID NO: 17, or (b) the complement of the nucleic acid molecule of (a).  
10
3. The isolated nucleic acid of claim 1, wherein the GPI anchor sequence is absent or substituted and inactive.
4. The nucleic acid of claim 1, wherein the nucleic acid has at least 75% sequence identity to (a) a nucleic acid molecule encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 27 to 400 of SEQ ID NO: 15 or the sequence of amino acids 27 to 369 of SEQ ID NO: 17, or (b) the complement of the nucleic acid molecule of (a).  
15
5. The isolated nucleic acid of claim 1, comprising a nucleic acid encoding a GFR $\alpha$ 3 polypeptide having amino acid residues 27 to 400 of SEQ ID NO: 15 or 27 to 369 of SEQ ID NO: 17.
- 20 6. The isolated nucleic acid of claim 1 comprising DNA encoding a GFR $\alpha$ 3 polypeptide having amino acid residues 1 to 400 of SEQ ID NO: 15 or residues 1 to 369 of SEQ ID NO: 17.
7. An isolated nucleic acid comprising nucleic acid having at least a 65% sequence identity to (a) a nucleic acid molecule encoding the same mature polypeptide encoded by the cDNA in ATCC Deposit No. 209752 (designation: DNA48613-1268) or in ATCC Deposit No. 209751, or (b) the complement of the DNA molecule of (a) or (b).  
25
8. The isolated nucleic acid of claim 7, comprising the GFR $\alpha$ 3 encoding sequence of the cDNA in ATCC deposit No. 209752 (designation: DNA48613-1268), in ATCC Deposit No. 209571, or a sequence which hybridizes thereto under stringent conditions.
- 30 9. An isolated nucleic acid comprising a nucleic acid having at least a 65% sequence identity to (a) a nucleic acid molecule encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 84 to 360 of

SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20, or (b) the complement of the nucleic acid molecule of (a).

10. The isolated nucleic acid of claim 9, comprising a GFR $\alpha$ 3 encoding sequence which hybridizes under stringent conditions to (a) a nucleic acid molecule encoding a GFR $\alpha$ 3 polypeptide comprising the sequence of amino acids 84 to 360 of SEQ ID NO: 15, amino acids 84 to 329 of SEQ ID NO: 17, or the sequence of amino acids 110 to 386 of SEQ ID NO: 20, or (b) the complement of the nucleic acid molecule of (a).
11. A vector comprising the nucleic acid of claim 1.
12. The vector of claim 9 operably linked to control sequences recognized by a host cell transformed with the vector.
- 10 13. A host cell comprising the vector of claim 10.
14. The host cell of claim 11 wherein said cell is a CHO cell, an *E. coli.*, or a yeast cell.
15. A process for producing GFR $\alpha$ 3 polypeptides comprising culturing the host cell of claim 11 under conditions suitable for expression of GFR $\alpha$ 3 and recovering GFR $\alpha$ 3 from the cell culture.
- 15 16. A polypeptide comprising a sequence having at least 65% sequence identity with amino acid residues 84 to 360 of SEQ ID NO: 15 or 84 to 329 of SEQ ID NO: 17.
17. The polypeptide of claim 16 that is an isolated native sequence GFR $\alpha$ 3 polypeptide.
18. The polypeptide of claim 16 with its GPI anchor sequence absent or substituted and inactive.
19. The polypeptide of claim 16 comprising amino acid residues 27 to 400 of SEQ ID NO: 15, amino acid residues 27 to 369 of SEQ ID NO: 17, amino acid residues 84 to 360 of SEQ ID NO: 15, or amino acid residues 110 to 386 of SEQ ID NO: 20.
- 20 20. A chimeric molecule comprising a GFR $\alpha$ 3 polypeptide fused to a heterologous amino acid sequence.
21. The chimeric molecule of claim 20 wherein said heterologous amino acid sequence is an epitope tag sequence.
22. The chimeric molecule of claim 20 wherein said heterologous amino acid sequence is a Fc region of an immunoglobulin.
- 25

23. An antibody which specifically binds to GFR $\alpha$ 3 polypeptide.
24. The antibody of claim 23 that is an agonist antibody.
25. The use of the antibody of claim 23 to treat a neuronal disorder of the periphery.
26. A method for measuring agonist binding to a polypeptide comprising an agonist-binding domain of  
5 an  $\alpha$ -subunit receptor, comprising the steps of exposing the polypeptide positioned in a cell membrane to a candidate agonist and measuring homo-dimerization or homo-oligomerization of the polypeptide.
27. The method of claim 26, wherein the  $\alpha$ -subunit receptor is a GFR $\alpha$ -receptor.
28. The method of claim 26, wherein the polypeptide further comprises a dimerization- or  
10 oligomerization-activated enzymatic domain and homo-dimerization or homo-oligomerization is detected by measuring enzymatic activity of the polypeptide.
29. The method of claim 28, wherein the enzymatic domain is the intracellular autocatalytic domain of a receptor tyrosine kinase and homo-dimerization or homo-oligomerization is detected by measuring autophosphorylation of the polypeptide.
30. A method of measuring autophosphorylation of a polypeptide receptor construct comprising a ligand-  
15 binding domain of an  $\alpha$ -subunit receptor, the intracellular catalytic domain of a tyrosine kinase receptor, and a flag epitope, comprising the steps of:
- (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein, positioned in their membranes, the cells have the polypeptide receptor construct;
  - 20 (b) exposing the adhering cells to an analyte;
  - (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
  - (d) coating a second solid phase with a capture agent which binds specifically to the flag epitope so that the capture agent adheres to the second solid phase;
  - 25 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the receptor construct adheres to the second solid phase;
  - (f) washing the second solid phase so as to remove unbound cell lysate;
  - (g) exposing the adhering receptor construct to an anti-phosphotyrosine antibody which identifies phosphorylated tyrosine residues in the tyrosine kinase receptor; and
  - (h) measuring binding of the anti-phosphotyrosine antibody to the adhering receptor construct.



31. The method of claim 30 wherein the cells are transformed with nucleic acid encoding the receptor construct prior to step (a).
32. The method of claim 30 wherein the cells comprise a mammalian cell line.
33. The method of claim 30 wherein the cells are adherent.
- 5 34. The method of claim 30 wherein the capture agent comprises a capture antibody.
35. The method of claim 30 wherein the first solid phase comprises a well of a first assay plate.
36. The method of claim 30 wherein the anti-phosphotyrosine antibody is labelled.
37. The method of claim 36 wherein the label comprises an enzyme which is exposed to a color reagent and the color change of the color reagent is determined in step (h).
- 10 38. The method of claim 30 wherein the flag polypeptide is fused to the amino terminus of the  $\alpha$ -subunit receptor ligand-binding domain.
39. The method of claim 30 wherein the flag polypeptide is fused to the carboxyl terminus of the tyrosine kinase receptor intracellular catalytic domain.
40. The method of claim 30 wherein the tyrosine kinase receptor is a Rse receptor, a trk A receptor, a trk B receptor or a trk C receptor.
- 15 41. The method of claim 30 wherein the  $\alpha$ -subunit receptor is a GFR $\alpha$ -receptor.
42. The method of claim 40 wherein the receptor construct further comprises the transmembrane domain of the Rse receptor and the flag epitope comprises the gD polypeptide.
43. The method of claim 30 wherein the analyte comprises an agonist for the  $\alpha$ -subunit receptor.
- 20 44. The method of claim 30 wherein the analyte comprises an antagonist for the  $\alpha$ -subunit receptor.
45. The method of claim 44 wherein the antagonist competitively inhibits binding or activation of the  $\alpha$ -subunit receptor by an agonist thereto and step (b) is followed by a step wherein the adhering cells are exposed to the agonist.

46. The method of claim 30 wherein the analyte is a composition which comprises an antagonist and an agonist for the  $\alpha$ -subunit receptor and the assay measures the ability of the antagonist to bind to the agonist and thereby reduce activation of the polypeptide construct by the agonist.
47. A method for measuring autophosphorylation of a polypeptide receptor construct comprising a ligand-binding domain of an  $\alpha$ -subunit receptor, the intracellular catalytic domain of a tyrosine kinase receptor, and a flag epitope, comprising the steps of:
- (a) coating a well of a first assay plate with a homogeneous population of adherent cells so that the cells adhere to the well, wherein the cells have the polypeptide receptor construct positioned in the cell membranes thereof;
  - (b) exposing the adhering cells to an analyte;
  - (c) solubilizing the adhering cells thereby releasing cell lysate therefrom;
  - (d) coating a well of a second assay plate with a capture agent which binds specifically to the polypeptide receptor construct so that the capture agent adheres to the well;
  - (e) exposing the cell lysate obtained in step (c) to the adhering capture agent so that the polypeptide receptor construct adheres to the well;
  - (f) washing the well so as to remove unbound cell lysate;
  - (g) exposing the adhering polypeptide receptor construct to an anti-phosphotyrosine antibody which binds selectively to phosphorylated tyrosine residues in the polypeptide receptor construct;
  - (h) measuring binding of the anti-phosphotyrosine antibody to the adhering polypeptide receptor construct.
48. The method of claim 47 wherein the  $\alpha$ -subunit receptor is a GFR $\alpha$ -receptor.
49. A polypeptide comprising an  $\alpha$ -subunit receptor ligand-binding domain, a flag polypeptide, and an intracellular catalytic domain of a tyrosine kinase receptor.
50. The polypeptide of claim 49, wherein the flag polypeptide comprises the gD flag epitope.
51. The polypeptide of claim 49, wherein the tyrosine kinase receptor is a Rse receptor.
52. The polypeptide of claim 51 further comprising the transmembrane domain of the Rse receptor.
53. The polypeptide of claim 49, wherein the  $\alpha$ -subunit receptor is a GFR $\alpha$  receptor.

54. A kit comprising a solid phase coated with a capture agent which binds specifically to a flag polypeptide, and a polypeptide comprising an  $\alpha$ -subunit receptor ligand-binding domain, a flag polypeptide, and an intracellular catalytic domain of a tyrosine kinase receptor.
55. The kit of claim 54 wherein the solid phase comprises a well of a microtiter plate.
- 5 56. The kit of claim 54 further comprising a labeled anti-phosphotyrosine antibody.
57. The kit of claim 56 wherein the label comprises an enzyme.
58. The kit of claim 54 further comprising a cell transformed with a nucleic acid encoding a polypeptide comprising an  $\alpha$ -subunit receptor ligand-binding domain, a flag polypeptide, and an intracellular catalytic domain of a tyrosine kinase receptor.
- 10 59. An assay for measuring phosphorylation of polypeptide receptor construct comprising a ligand-binding domain of an  $\alpha$ -subunit receptor, the intracellular catalytic domain of a kinase receptor, and a flag epitope, comprising the steps of:
  - (a) coating a first solid phase with a homogeneous population of eukaryotic cells so that the cells adhere to the first solid phase, wherein the cells comprise the polypeptide receptor construct;
  - 15 (b) exposing the adhering cells to an analyte;
  - (c) solubilizing the adhering cells, thereby releasing cell lysate therefrom;
  - (d) coating a second solid phase with a capture agent which binds specifically to the flag polypeptide so that the capture agent adheres to the second solid phase;
  - 20 (e) exposing the adhering capture agent to the cell lysate obtained in step (c) so that the receptor construct adheres to the second solid phase;
  - (f) washing the second solid phase so as to remove unbound cell lysate;
  - (g) exposing the adhering kinase construct to an antibody which identifies phosphorylated residues in the receptor construct; and
  - 25 (h) measuring binding of the antibody to the adhering receptor construct.
60. The assay of claim 59 wherein the  $\alpha$ -receptor is a GFR $\alpha$ -receptor.
61. The assay of claim 59 wherein the kinase receptor is a serine-threonine kinase receptor.
62. The assay of claim 59 which measures phosphatase activity.

63. The assay of claim 62 wherein the cells further comprise a phosphatase and the assay further comprises the step of exposing the eukaryotic cells to a phosphatase inhibitor prior to step (c).
64. The assay of claim 62 which further comprises the steps in between steps (f) and (g) of exposing the adhering kinase construct to a phosphatase and then washing the second solid phase so as to remove unbound phosphatase.
- 5

1	GAATTGGCC	CTCGAGGCCA	AGAATTCGGC	ACGAGGCGCG	GCGCCACAGG	CAGGCAGAGC	GCTGTGCGAT	CCCGGGCGTC	CACCCGCCAT	GGGGCTCTCC	
1	CTTAAACCGG	GAGCTCCGGT	TCCTAAGCCG	TGCTCCGCGC	CGCGGTCGC	GTCCGTCTCG	CGACAGCGTA	GGGCCCGCAG	GTGGCGGTA	CCCGAGAGG	Me tGlyLeuSer
101	TGGAGCCCGC	GACCTCCACT	GCTGATGATC	CTGCTACTGG	TGCTGTCTGT	GTGGTGCCA	CTTGGAGCAG	GAACTCCCT	TGCCACAGAG	AACAGTTTG	
5	ACCTCGGGG	CTGGAGGTGA	CGACTACTAG	GACGATGACC	ACGACAGCAA	CACCGACGGT	GAACCTCGTC	CTTTGAGGGA	ACGGTGTCTC	TTGTCCAAAC	
5	TrpSerProA	rgProProLe	uLeuMetIle	LeuLeuLeuV	alleuSerLe	uTrpLeuPro	LeuGlyAlaG	lyAsnSerLe	uAlaThrGlu	AsnArgPheVal	
201	TGAACAGCTG	TACCCAGGCC	AGAAAGAAAT	GCGAGGCTAA	TCCCGCTTGC	AAGGCTGCCT	ACCAGCACCT	GGGCTCCTGC	ACCTCCAGTT	TAAGCAGGCC	
39	AsnSerCy	sThrGlnAla	ArgLysLysC	ysGluAlaAs	nProAlaCys	LysAlaAlaT	yrGlnHisLe	uGlySerCys	ThrSerSerL	euSerArgPro	
301	GCTGCCCTTA	GAGGAGTCTG	CCATGTCTGC	AGACTGCCTA	GAGGCAGCAG	AACAACTCAG	GAACAGCTCT	CTGATAGACT	GCAGGTGCCA	TCGGCGCATG	
72	LeuProLeu	GluGluSerA	laMetSerAl	laMetSerAl	aAspCysLeu	GluAlaAlaG	luGlnLeuAr	gAsnSerSer	LeuIleAspC	ysArgCysHi	sArgArgMet
401	AAGACCAAG	CTACCTGTCT	GGACATTTAT	TGGACCGTTC	ACCCTGCCCG	AAGCCTTGGT	GACTACGAGT	TGGATGTCTC	ACCCTATGAA	GACACAGTGA	
105	TTCTGTTTC	GATGGACAGA	CCGTAAATA	ACCTGGCAAG	TGGACGGGC	TTCCGGAACCA	CTGATGCTCA	ACCTACAGAG	TGGGATACTT	CTGTGTCACT	
501	CCAGCAAACC	CTGGAAAATG	AATCTTAGCA	AGTTGAACAT	GCTCAAACCA	GACTCGGACC	TCTGCCTCAA	ATTTGCTATG	CTGTGTACTC	TTCACGACAA	
139	GGTCGTTGG	GACCTTTTAC	TTAGAAATCGT	TCAACTTGTA	CGAGTTTGGT	CTGAGCCCTGG	AGACGGAGTT	TAAACGATAC	GACACATGAG	AAGTGTGTT	
601	GTGTGACCGC	CTGGCGAAGG	CCTACGGGGA	GGCATGCTCA	GGGATCCGT	GCCAGCGCCA	CCTCTGCCCTA	GCCCAGCTGC	GCTCCTTCTT	TGAGAAGGCA	
172	CysAspArg	LeuArgLysA	laTyrGlyG1	uAlaCysSer	GlyIleArgC	ysGlnArgHi	sLeuCysLeu	AlaGlnLeuA	rgSerPhePh	eGluLysAla	
701	GCAGAGTCCC	ACGCTCAGGG	TCTGCTGCTG	TGTCCCTGTG	CACCAGAAGA	TGCGGGCTGT	GGGAGCGGC	GCGTAAACAC	CATCGCCCCC	AGTTGCGCCC	
205	AlaGluSerH	isAlaGlnG1	yLeuLeuLeu	CysProCysA	laProGluAs	pAlaGlyCys	GlyGluArgA	rgArgAsnTh	rIleAlaPro	SerCysAlaLeu	
801	TGCTTCTGT	AACCCCAAT	TGCCTGGATC	TGCGGAGCTT	CTGCCGTGGC	GACCCTTGT	GCAGATCAGC	CCTGATGGAC	TTCCAGACCC	ACTGTCTATC	
239	ProSerVa	lThrProAsn	CysLeuAspL	euArgSerPh	eCysArgAla	AspProLeuC	ysArgSerAr	gLeuMetAsp	PheGlnThrH	isCysHisPro	
901	TATGGACATC	CTTGGGACTT	GTGCAACTGA	GCAGTCCAGA	TGTCTGCGG	CATACCTGGG	GCTGATTGGG	ACTGCCATGA	CCCCAAACTT	CATCAGCAAG	
272	MetAspIle	LeuGlyThrc	ysAlaThrG1	uGlnSerArg	CysLeuArgA	laTyrLeuG1	yLeuIleGly	ThrAlaMetT	hrProAsnPh	eIleSerLys	

FIG. 1A

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 CAGTTGTGAT GACAACGGAA TTCGACGTGG ACGGCTCCGT CGCGTGTGGA TGTCCTGCTC ACCTTTCCAG GAAGAGGGTC TTGGGACGG  
 305 ValAsnThrT hrValAlaLe uSerCysThr CysArgGlyS erGlyAsnLe uGlnAspGlu CysGluGlnL euGluArgSe rPheSerGln AsnProCysLeu  
 1101 TCGTGGAGGC CATTGCAGCT AAGATGCGTT TCCACAGACA GCTCTTCTCC CAGGACTGGG CAGACTCTAC TTTTTCAGTG GTGCAGCAGC AGAACAGCAA  
 AGCAGCTCCG GTAACGTCGA TTCTACGCAA AGGTGTCTGT CGAGAAGAGG GTCCTGACCC GTCTGAGATG AAAAAGTCAC CACGTCGTCG TCTTGTCTGT  
 339 ValGluAl aileAlaAla LysMetArgP heHisArgG1 nLeuPheSer GlnAspTrpA laAspSerTh rPheSerVal ValGlnGlnG InAsnSerAsn  
 1201 CCCTGCTCTG AGACTGCAGC CCAGGCTACC CATTCCTTCTT TTTCTCCATCC TTCCCTTGAT TCTGCTGCAG ACCCTCTGGT AGCTGGGCTT COTCAGGGTC  
 GGGACGAGAC TCTGACGTCTG GGTCCGATGG GTAAGAAAGA AAGAGGTAGG AAGGGAATA AGACGACGTC TGGGAGACCA TCGACCCCGAA GGAGTCCCAG  
 372 ProAlaLeu ArgLeuGlnP roArgLeuPr oileLeuSer PheSerIleL euProLeuI eLeuLeuGln ThrLeuTrp  
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 GAAACAGGAG AGGTGGTGTG GGTCTGACTA AACGTCGGAC ACCACCTCTCT CTTGAGCGGT CGGACACCTT CTTCCTGCGTC GCACGATGTG TCGTTGGGCC  
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 TTGGTTGGTC CGTAAGGCGT CGTGAGGGC AGACGAGTCC TTCTCCAGAA TCTTCACTCC CGACACTGGG AAGGCTAGGA CTCGCCGATC AAAAGTTTGG  
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 TTAGGACTTG TAAACCCGTA CTTCTCGATT TCAGAAACCC AGAACAAATT GAGGATAATG ACAGGGGTTT AAGGGGATCA GGAACCCAG TACTAATTTG  
 1901 ATTTTGACTT AAAAAAAA AAAAAAAA AAAAAA  
 TAAACCTGAA TTTTTTTTT TTTTTTTTT TTTTTT

FIG. 1B

Sequence logo for the 3' UTR of the rGFRα1 gene. The logo shows the conservation of nucleotides across 1000 bp upstream of the start codon. The y-axis represents the information content in bits, ranging from 0 to 4. The x-axis shows the position relative to the start codon, from -1000 to 0. The logo is divided into three regions: the 5' UTR (positions -1000 to -500), the coding region (positions -500 to 0), and the 3' UTR (positions 0 to 1000). The 5' UTR shows high conservation of the start codon (ATG) and the 5' cap (m7Gppp). The coding region shows high conservation of the start codon (ATG) and the 5' cap (m7Gppp). The 3' UTR shows high conservation of the stop codon (TGA) and the 3' cap (m7Gppp).

hGFra3	1	M	V	R	P	L	N	P	R	P	L	P	P	V	V	L	M	L	L	L	L	P	P	S	P	L	P	L	A	G	D	P	L	P	T	E	S	R	L	M	N	S	C	L	Q	A	R	R	K		
mGFra3	1	.	.	.	M	G	L	S	W	S	P	R	P	P	L	L	M	I	L	L	L	V	L	S	L	W	.	L	P	L	G	A	G	N	S	L	A	T	E	N	R	F	V	N	S	C	T	Q	A	R	K

hGFra3	51	C	Q	A	D	P	T	C	S	A	Y	H	L	D	S	C	T	S	S	I	S	T	P	L	P	S	E	E	P	S	V	P	A	D	C	L	E	A	A	Q	Q	L	R	N	S	S	L	I	G		
mGFra3	48	C	E	A	N	P	A	C	K	A	A	Y	Q	H	L	G	S	C	T	S	S	L	S	R	P	L	P	L	E	E	S	A	M	S	A	D	C	L	E	A	A	E	Q	L	R	N	S	S	L	I	D

hGFra3	101	C	M	C	H	R	R	M	K	N	Q	V	A	C	L	D	I	Y	W	T	V	H	R	A	R	S	L	G	N	Y	E	L	D	V	S	P	Y	E	D	T	V	T	S	K	P	W	K	M	N	L	S
mGFra3	98	C	R	C	H	R	R	M	K	H	Q	A	T	C	L	D	I	Y	W	T	V	H	P	A	R	S	L	G	D	Y	E	L	D	V	S	P	Y	E	D	T	V	T	S	K	P	W	K	M	N	L	S

hGFra3	151	K	L	N	M	L	K	P	D	S	D	L	C	L	K	F	A	M	L	C	T	L	N	D	K	C	D	R	L	R	K	A	Y	G	E	A	C	S	G	P	H	C	Q	R	H	V	C	L	R	Q	L
mGFra3	148	K	L	N	M	L	K	P	D	S	D	L	C	L	K	F	A	M	L	C	T	L	H	D	K	C	D	R	L	R	K	A	Y	G	E	A	C	S	G	I	R	C	Q	R	H	L	C	L	A	Q	L

hGFra3	201	L	T	F	F	E	K	A	A	E	P	H	A	Q	G	L	L	C	P	C	A	P	N	D	R	G	C	G	E	R	R	R	N	T	I	A	P	N	C	A	L	P	P	V	A	P	N	C	L	E
mGFra3	198	R	S	F	F	E	K	A	A	E	S	H	A	Q	G	L	L	C	P	C	A	P	E	D	A	G	C	G	E	R	R	R	N	T	I	A	P	S	C	A	L	P	S	V	T	P	N	C	L	D

hGFra3	251	L	R	R	L	C	F	S	D	P	L	C	R	S	R	L	V	D	F	Q	T	H	C	H	P	M	D	I	L	G	T	C	A	T	E	Q	S	R	C	L	R	A	Y	L	G	L	I	G	T	A	M
mGFra3	248	L	R	S	F	C	R	A	D	P	L	C	R	S	R	L	M	D	F	Q	T	H	C	H	P	M	D	I	L	G	T	C	A	T	E	Q	S	R	C	L	R	A	Y	L	G	L	I	G	T	A	M

hGFra3	301	T	P	N	F	V	S	N	V	N	T	S	V	A	L	S	C	T	C	R	G	S	G	N	L	Q	E	E	C	E	M	L	E	G	F	F	S	H	N	P	C	L	T	E	A	I	A	A	K	M	R
mGFra3	298	T	P	N	F	I	S	K	V	N	T	T	V	A	L	S	C	T	C	R	G	S	G	N	L	Q	E	C	E	Q	L	E	R	S	F	S	Q	N	P	C	L	V	E	A	I	A	A	K	M	R	

hGFra3	351	F	H	S	Q	L	F	S	Q	D	W	P	H	P	T	F	A	V	M	A	H	Q	N	E	N	P	A	V	R	P	Q	P	W	V	P	S	L	F	S	C	T	L	P	L	I	L	L	S	L	W
mGFra3	348	F	H	R	Q	L	F	S	Q	D	W	A	D	S	T	F	S	V	V	Q	Q	N	S	N	P	A	L	R	L	Q	P	R	L	P	I	L	S	F	S	I	L	P	L	I	L	L	Q	T	L	W

FIG. 3



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48613	1	MVRPLNPRPLPPVVLMLLLPPSPPLPLAAGDPLPTESRLMNSCLQARRK	
48614	1	MVRPLNPRPLPPVVLMLLLPPSPPLPLAAGDPLPTESRLMNSCLQARRK	
48613	51	CQADPTCSAAYHHLDSCTSSISTPLPSEEPSVPADCLEAAQQLRNSSLIG	
48614	51	CQADPTCSAAYHHLDSCTSSISTPLPSEEPSVPADCLEAAQQLRNSSLIG	
48613	101	CMCHRRMKNQVACLDIYWTVHRRARSL	GNYELDVSPYEDT VTSKPKWMNLS
48614	101	CMCHRRMKNQVACLDIYWTVHRRARSL	
48613	151	KLNMLKPD	DSDLCCLKFAMLC TLNDKCDRLRKAYGEACSGPHCQRHVCLRQL
48614	127	-----	DSDLCCLKFAMLC TLNDKCDRLRKAYGEACSGPHCQRHVCLRQL
48613	201	LTFFEKAAEPHAQGLLLCPCAPNDRGCGERRRNTIAPNCALPPVAPNCLE	
48614	170	LTFFEKAAEPHAQGLLLCPCAPNDRGCGERRRNTIAPNCALPPVAPNCLE	
48613	251	LRRLCFSDPLCRSRLVDFQTHCHPMDILGTCA TEQSRCLRAYLGLGTAM	
48614	220	LRRLCFSDPLCRSRLVDFQTHCHPMDILGTCA TEQSRCLRAYLGLGTAM	
48613	301	TPNFVSNVNTSVALSCTCRGSGNLQEECEMLEGFFSHNPCLTEAIAAKMR	
48614	270	TPNFVSNVNTSVALSCTCRGSGNLQEECEMLEGFFSHNPCLTEAIAAKMR	
48613	351	FHSQLFSQDWPHPPTFAVMAHQENPAVRPQWPVPSLFSCTLPLILLLSLW	
48614	320	FHSQLFSQDWPHPPTFAVMAHQENPAVRPQWPVPSLFSCTLPLILLLSLW	

FIG. 4

FIG. 5A



**SUBSTITUTE SHEET (RULE 26)**

DNA48613.orf 1053 T C A C A G C C A A C T C T T C T C C C A G G A C T G C C A C C C T A C C T T G C T G T G A  
 GDNFRa1.orf 1029 C T C G A T G T G A C C G T G T G C A G C C T T C C C A G T A C A G A C C A C C A C T G  
 GDNFRa2.orf 1074 C A C G G A C C G T G A C C G T G T C C C C A A A G G C C C C T C G T T C A G G C C A C C C A G G

DNA48613.orf 1103 T G G C A C A C A G A A T G A A A C C C T G C T G T G A G G C C A C A G C C C T G G G T G C C C  
 GDNFRa1.orf 1079 C C A C T A C C A C A C T G C C C T C C G G T T A A G A A C A A C C C T G G G C C A G C A  
 GDNFRa2.orf 1124 C C C C T C G G G T G A G A A G A C G C C T T C T T G C C A G A T G A C C T C A G T G A C A G T

DNA48613.orf 1153 T C T C T T T T C T C C T G C A C G C T T C C C T T G A T T C T G C T C C T G A G C C T A T G T A  
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DNA48613.orf 1203 G . . . . .  
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 GDNFRa2.orf 1224 G G G C T G A A G G C C A C A A C T C C A A A G A G T A A G C A T G T G C T T C A C A G A G C

GDNFRa1.orf 1229 T T T C C A A T G G T A A T T A T G A A A A G A A G G T C T C G G T G C T T C C A G C C A C A T A  
 GDNFRa2.orf 1274 T C A C G A C A A A T A T C A T C C C A G G G A G T A C A A G G T G A T C A A C C T A A C T C A

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GDNFRa1.orf 1329 G G T C C T G T G G T A A C C G C T C T G T C C A C C C T A T T A T C T T T A C A G A A A C A T  
 GDNFRa2.orf 1374 G A T G C T G A A C A G G C C T T G T A G . . . . .

GDNFRa1.orf 1379 C A T A G

FIG. 5D

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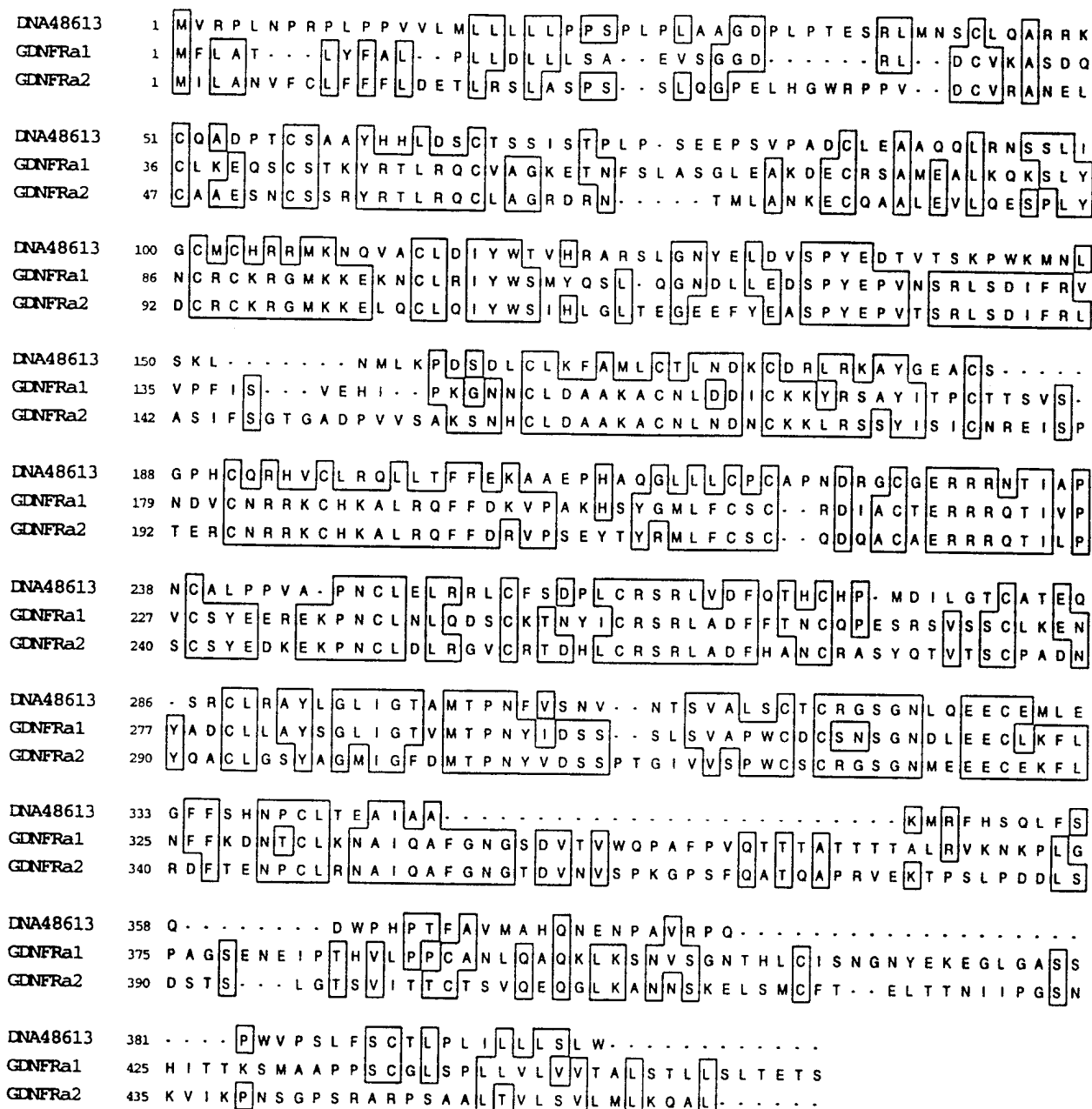


FIG. 6

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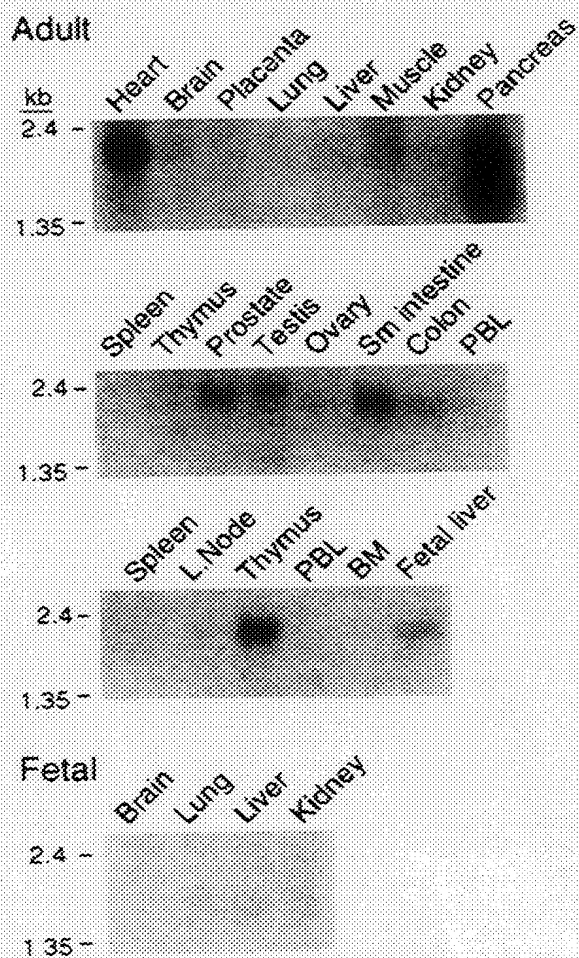


FIG. 7

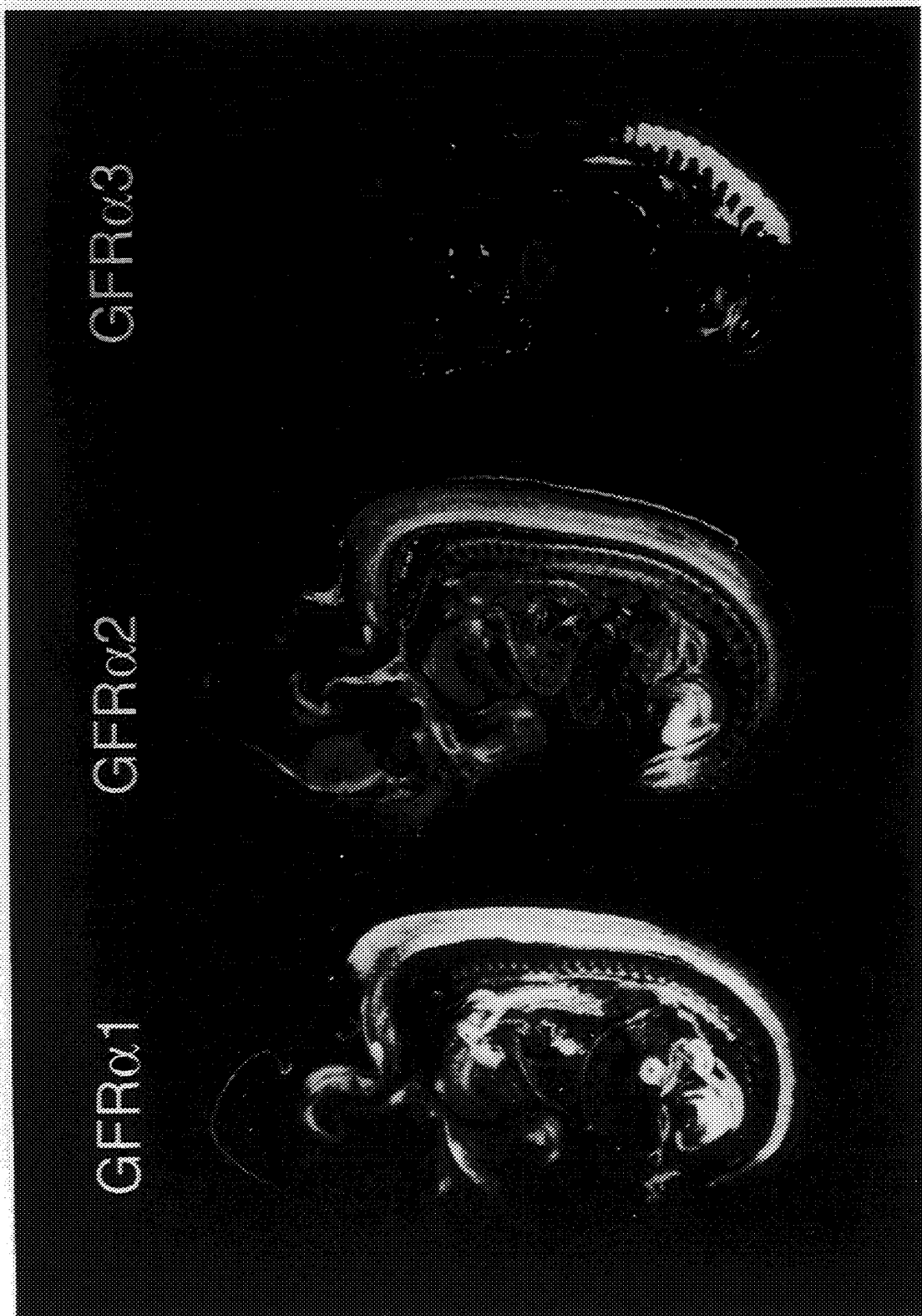
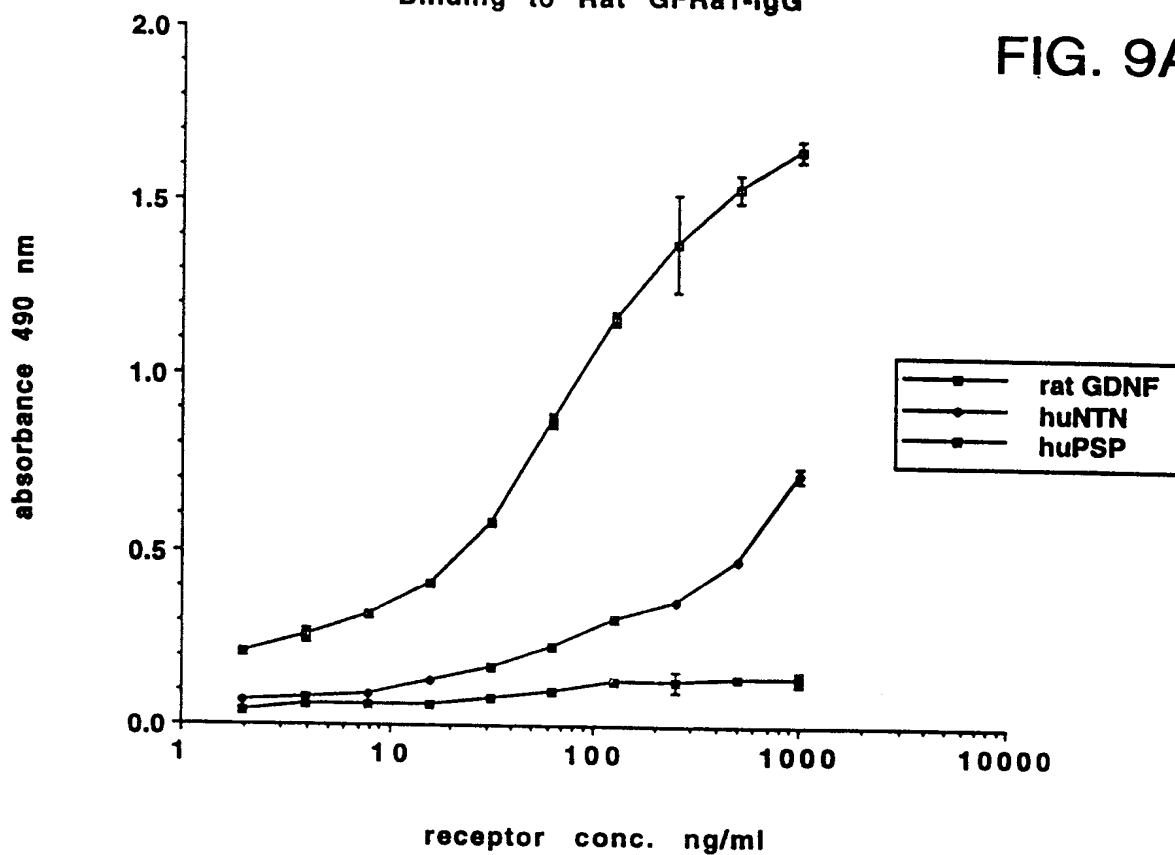


FIG. 8



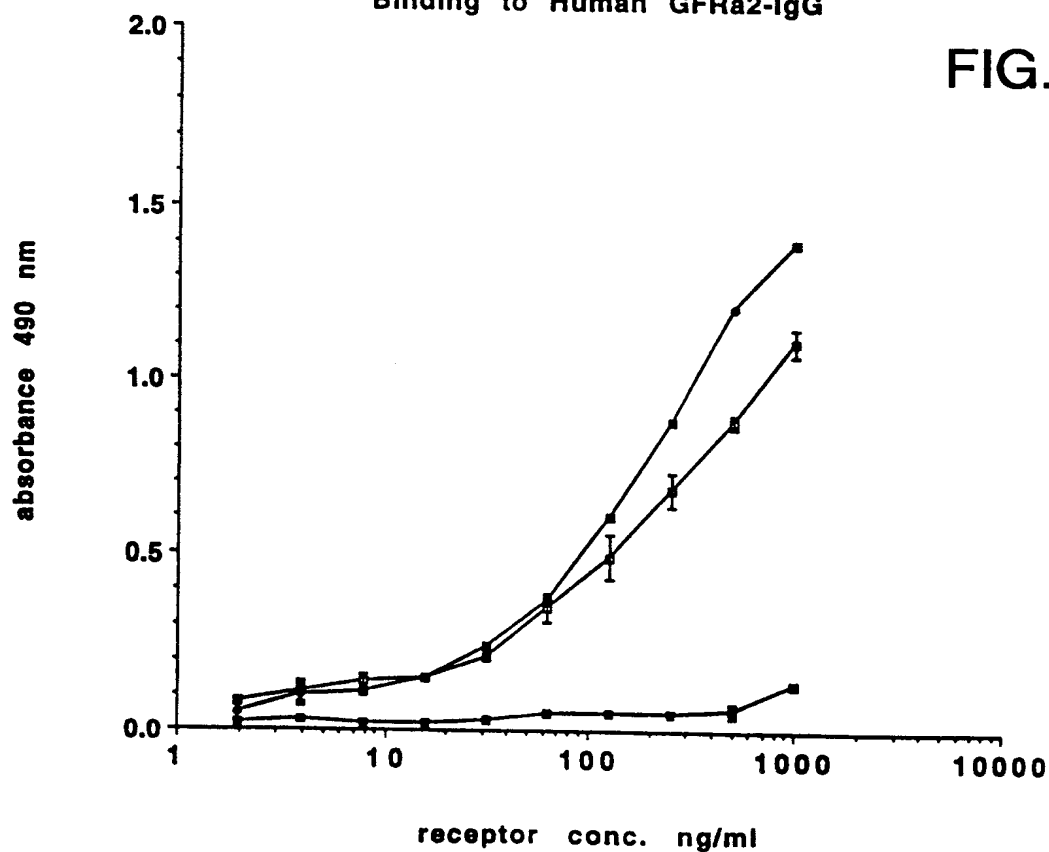
## Binding to Rat GFRa1-IgG

FIG. 9A

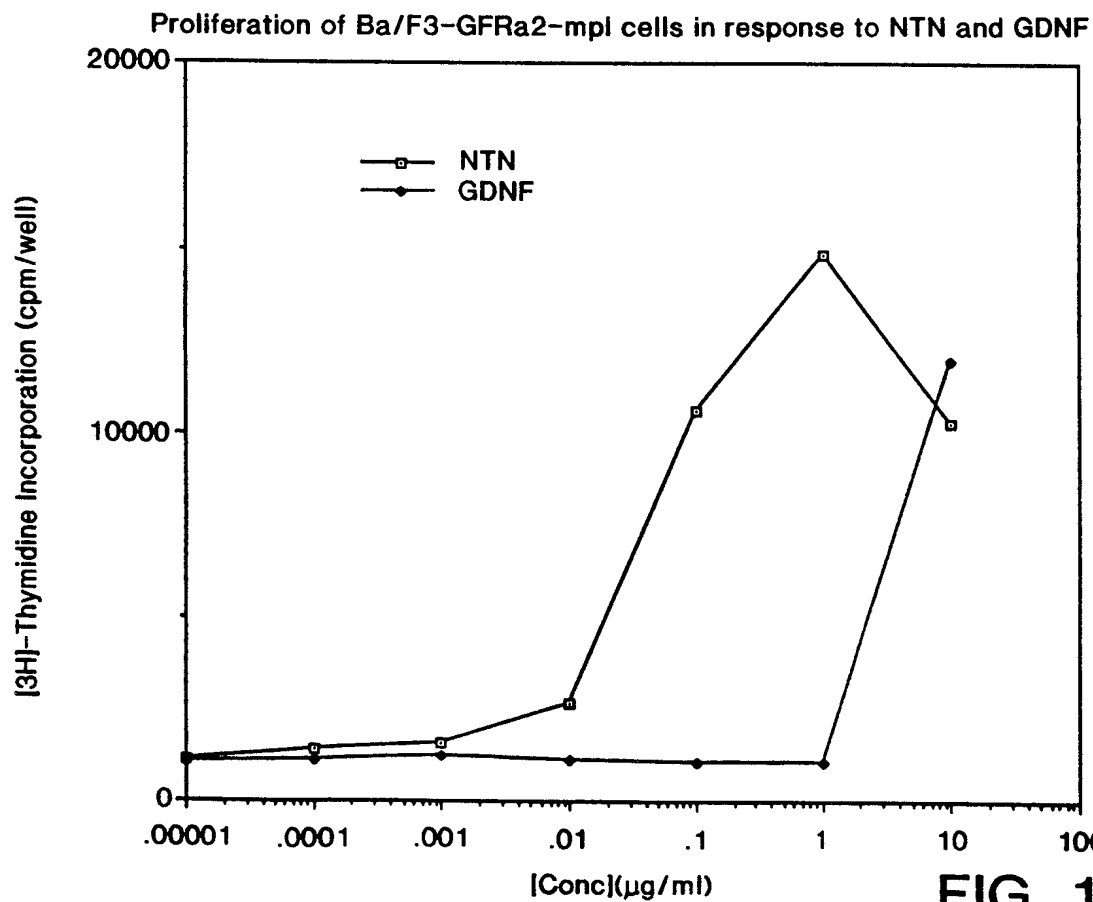
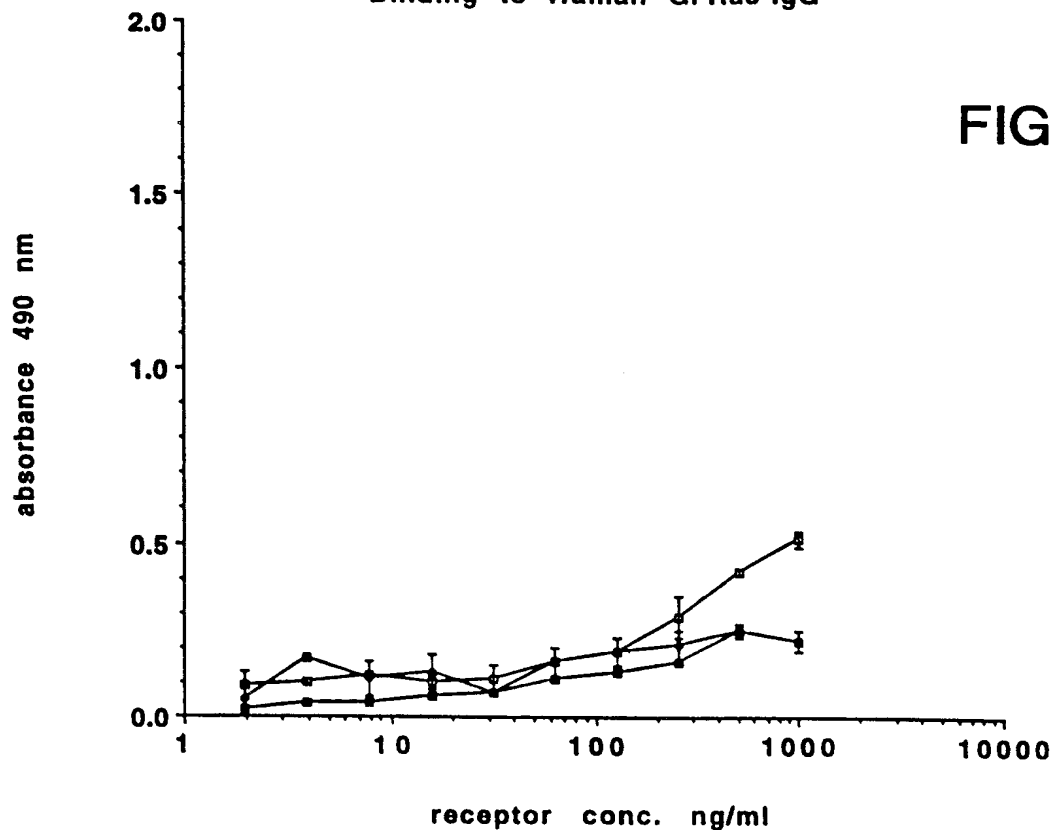


## Binding to Human GFRa2-IgG

FIG. 9B



## Binding to Human GFRa3-IgG

**FIG. 10**

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Phosphorylation of GFRa2-Rse in response to NTN

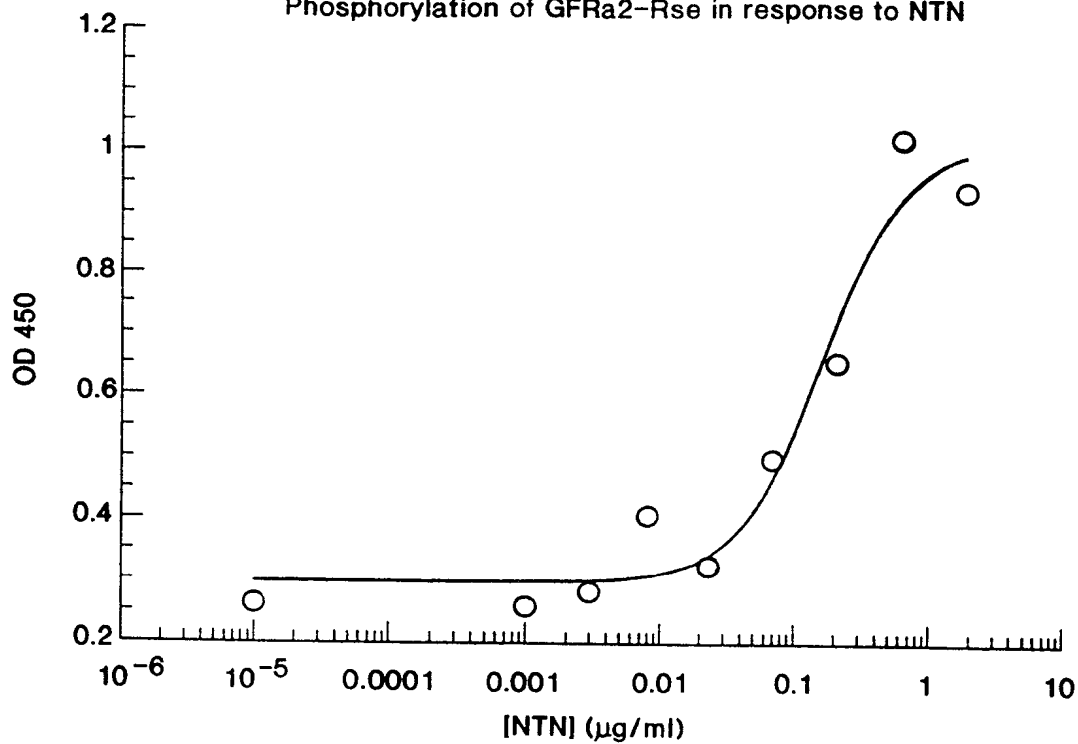


FIG. 11

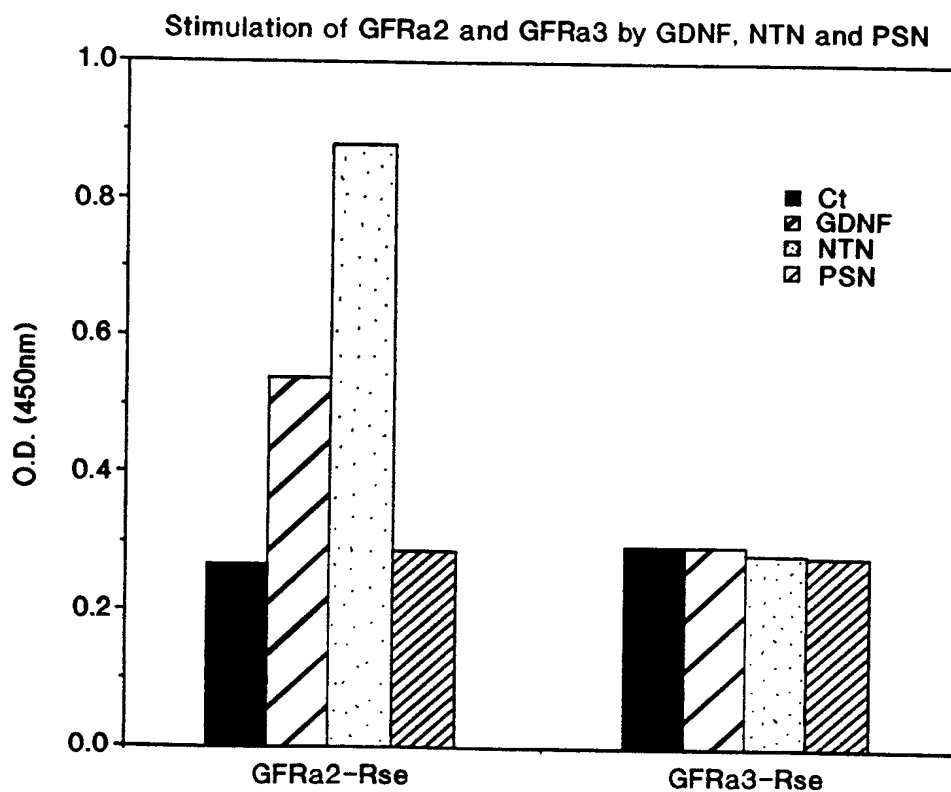


FIG. 12

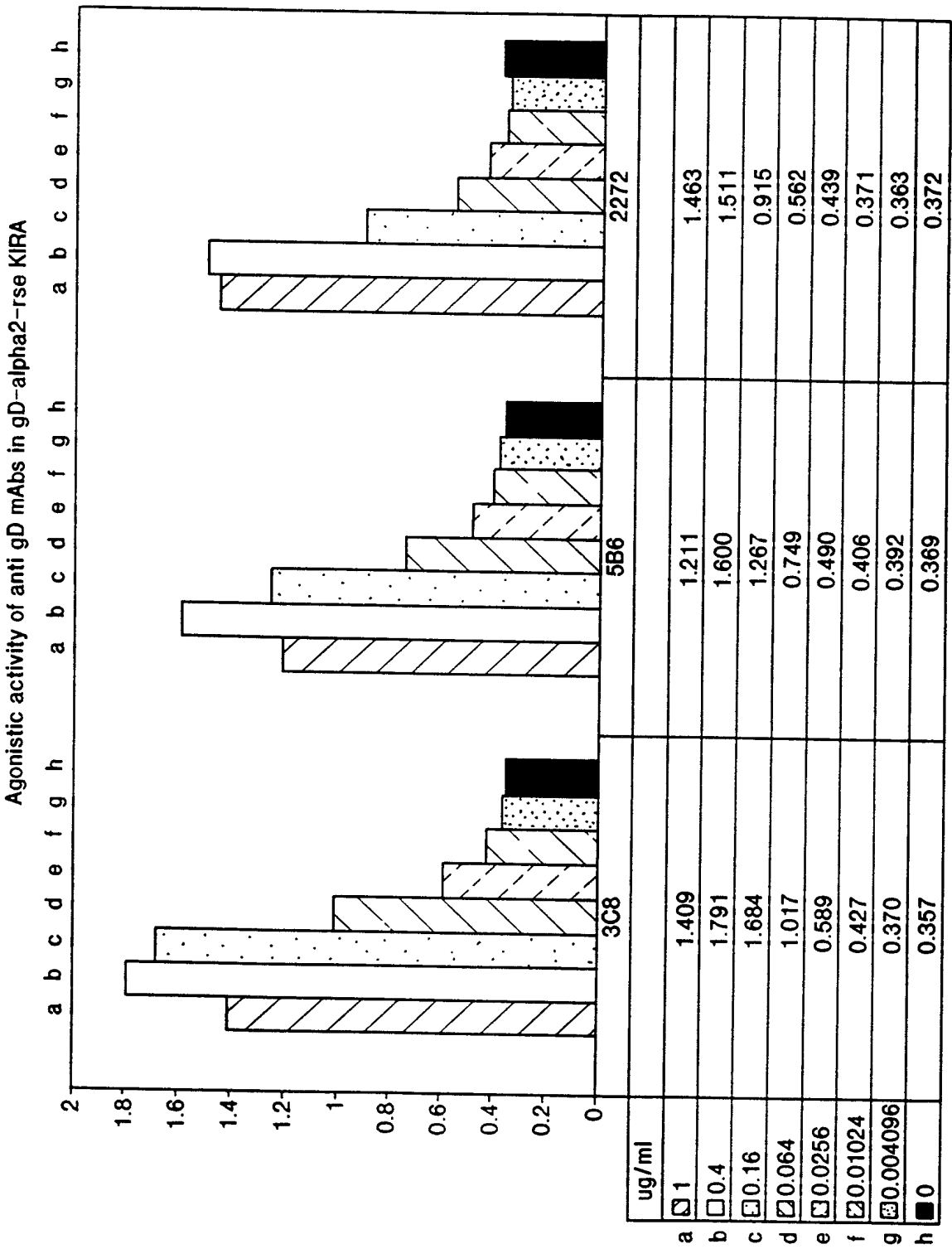


FIG. 13

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                20             25             30

10  Gly Ala Leu Leu Glu Pro Ala Thr Ser Thr Ala Asp Asp Pro Ala
    35             40             45

    Thr Gly Ala Val Val Val Ala Ala Thr Trp Ser Arg Lys Leu Pro
                50             55             60

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    Gln Thr Leu Glu Lys Ser Gln Val Glu His Ala Gln Thr Arg Leu
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	Gln Gly Pro Leu Ser Ser Pro Pro His	Pro Asp Phe Ala Ala Cys	
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    20 25 30

    Gly Asp Pro Leu Pro Thr Glu Ser Arg Leu Met Asn Ser Cys Leu
25 35 40 45

    Gln Ala Arg Arg Lys Cys Gln Ala Asp Pro Thr Cys Ser Ala Ala
    50 55 60

    Tyr His His Leu Asp Ser Cys Thr Ser Ser Ile Ser Thr Pro Leu
    65 70 75

30  Pro Ser Glu Glu Pro Ser Val Pro Ala Asp Cys Leu Glu Ala Ala
    80 85 90

    Gln Gln Leu Arg Asn Ser Ser Leu Ile Gly Cys Met Cys His Arg
    95 100 105

    Arg Met Lys Asn Gln Val Ala Cys Leu Asp Ile Tyr Trp Thr Val
35 110 115 120

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	His Arg Ala Arg Ser Leu Gly Asn Tyr Glu Leu Asp Val Ser Pro	125	130	135
	Tyr Glu Asp Thr Val Thr Ser Lys Pro Trp Lys Met Asn Leu Ser	140	145	150
5	Lys Leu Asn Met Leu Lys Pro Asp Ser Asp Leu Cys Leu Lys Phe	155	160	165
	Ala Met Leu Cys Thr Leu Asn Asp Lys Cys Asp Arg Leu Arg Lys	170	175	180
10	Ala Tyr Gly Glu Ala Cys Ser Gly Pro His Cys Gln Arg His Val	185	190	195
	Cys Leu Arg Gln Leu Leu Thr Phe Phe Glu Lys Ala Ala Glu Pro	200	205	210
	His Ala Gln Gly Leu Leu Leu Cys Pro Cys Ala Pro Asn Asp Arg	215	220	225
15	Gly Cys Gly Glu Arg Arg Arg Asn Thr Ile Ala Pro Asn Cys Ala	230	235	240
	Leu Pro Pro Val Ala Pro Asn Cys Leu Glu Leu Arg Arg Leu Cys	245	250	255
20	Phe Ser Asp Pro Leu Cys Arg Ser Arg Leu Val Asp Phe Gln Thr	260	265	270
	His Cys His Pro Met Asp Ile Leu Gly Thr Cys Ala Thr Glu Gln	275	280	285
	Ser Arg Cys Leu Arg Ala Tyr Leu Gly Leu Ile Gly Thr Ala Met	290	295	300
25	Thr Pro Asn Phe Val Ser Asn Val Asn Thr Ser Val Ala Leu Ser	305	310	315
	Cys Thr Cys Arg Gly Ser Gly Asn Leu Gln Glu Glu Cys Glu Met	320	325	330
30	Leu Glu Gly Phe Phe Ser His Asn Pro Cys Leu Thr Glu Ala Ile	335	340	345
	Ala Ala Lys Met Arg Phe His Ser Gln Leu Phe Ser Gln Asp Trp	350	355	360
	Pro His Pro Thr Phe Ala Val Met Ala His Gln Asn Glu Asn Pro	365	370	375
35	Ala Val Arg Pro Gln Pro Trp Val Pro Ser Leu Phe Ser Cys Thr	380	385	390
	Leu Pro Leu Ile Leu Leu Leu Ser Leu Trp	395	400	

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 cccctgaacc cgcgaccgct gccgcccgtg gtcttgatgt tgctgctgct 200  
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&lt;210&gt; 17

&lt;211&gt; 369

&lt;212&gt; PRT

15 &lt;213&gt; Homo sapiens

&lt;400&gt; 17

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20	Met	Leu	Leu	Leu	Leu	Leu	Pro	Pro	Ser	Pro	Leu	Pro	Leu	Ala	Ala
					20					25					30
	Gly	Asp	Pro	Leu	Pro	Thr	Glu	Ser	Arg	Leu	Met	Asn	Ser	Cys	Leu
					35					40					45
	Gln	Ala	Arg	Arg	Lys	Cys	Gln	Ala	Asp	Pro	Thr	Cys	Ser	Ala	Ala
					50					55					60
25	Tyr	His	His	Leu	Asp	Ser	Cys	Thr	Ser	Ser	Ile	Ser	Thr	Pro	Leu
					65					70					75
	Pro	Ser	Glu	Glu	Pro	Ser	Val	Pro	Ala	Asp	Cys	Leu	Glu	Ala	Ala
					80					85					90
30	Gln	Gln	Leu	Arg	Asn	Ser	Ser	Leu	Ile	Gly	Cys	Met	Cys	His	Arg
					95					100					105
	Arg	Met	Lys	Asn	Gln	Val	Ala	Cys	Leu	Asp	Ile	Tyr	Trp	Thr	Val
					110					115					120
	His	Arg	Ala	Arg	Ser	Leu	Asp	Ser	Asp	Leu	Cys	Leu	Lys	Phe	Ala
					125					130					135
35	Met	Leu	Cys	Thr	Leu	Asn	Asp	Lys	Cys	Asp	Arg	Leu	Arg	Lys	Ala
					140					145					150

	Tyr Gly Glu Ala Cys Ser Gly Pro His Cys Gln Arg His Val Cys	
	155	160 165
	Leu Arg Gln Leu Leu Thr Phe Phe Glu Lys Ala Ala Glu Pro His	
	170	175 180
5	Ala Gln Gly Leu Leu Leu Cys Pro Cys Ala Pro Asn Asp Arg Gly	
	185	190 195
	Cys Gly Glu Arg Arg Arg Asn Thr Ile Ala Pro Asn Cys Ala Leu	
	200	205 210
10	Pro Pro Val Ala Pro Asn Cys Leu Glu Leu Arg Arg Leu Cys Phe	
	215	220 225
	Ser Asp Pro Leu Cys Arg Ser Arg Leu Val Asp Phe Gln Thr His	
	230	235 240
	Cys His Pro Met Asp Ile Leu Gly Thr Cys Ala Thr Glu Gln Ser	
	245	250 255
15	Arg Cys Leu Arg Ala Tyr Leu Gly Leu Ile Gly Thr Ala Met Thr	
	260	265 270
	Pro Asn Phe Val Ser Asn Val Asn Thr Ser Val Ala Leu Ser Cys	
	275	280 285
20	Thr Cys Arg Gly Ser Gly Asn Leu Gln Glu Glu Cys Glu Met Leu	
	290	295 300
	Glu Gly Phe Phe Ser His Asn Pro Cys Leu Thr Glu Ala Ile Ala	
	305	310 315
	Ala Lys Met Arg Phe His Ser Gln Leu Phe Ser Gln Asp Trp Pro	
	320	325 330
25	His Pro Thr Phe Ala Val Met Ala His Gln Asn Glu Asn Pro Ala	
	335	340 345
	Val Arg Pro Gln Pro Trp Val Pro Ser Leu Phe Ser Cys Thr Leu	
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30	Pro Leu Ile Leu Leu Leu Ser Leu Trp	
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	Gly	Asp	Pro	Leu	Pro	Thr	Glu	Ser	Arg	Leu	Met	Asn	Ser	Cys	Leu	
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	Gln	Ala	Arg	Arg	Lys	Cys	Gln	Ala	Asp	Pro	Thr	Cys	Ser	Ala	Ala	
					50					55					60	
5	Tyr	His	His	Leu	Asp	Ser	Cys	Thr	Ser	Ser	Ile	Ser	Thr	Pro	Leu	
					65					70					75	
	Pro	Ser	Glu	Glu	Pro	Ser	Val	Pro	Ala	Asp	Cys	Leu	Glu	Ala	Ala	
					80					85					90	
10	Gln	Gln	Leu	Arg	Asn	Ser	Ser	Leu	Ile	Gly	Cys	Met	Cys	His	Arg	
					95					100					105	
	Arg	Met	Lys	Asn	Gln	Val	Ala	Cys	Leu	Asp	Ile	Tyr	Trp	Thr	Val	
					110					115					120	
	His	Arg	Ala	Arg	Ser	Leu	Gly	Asn	Tyr	Glu	Leu	Asp	Val	Ser	Pro	
					125					130					135	
15	Tyr	Glu	Asp	Thr	Val	Thr	Ser	Lys	Pro	Trp	Lys	Met	Asn	Leu	Ser	
					140					145					150	
	Lys	Leu	Asn	Met	Leu	Lys	Pro	Asp	Ser	Asp	Leu	Cys	Leu	Lys	Phe	
					155					160					165	
20	Ala	Met	Leu	Cys	Thr	Leu	Asn	Asp	Lys	Cys	Asp	Arg	Leu	Arg	Lys	
					170					175					180	
	Ala	Tyr	Gly	Glu	Ala	Cys	Ser	Gly	Pro	His	Cys	Gln	Arg	His	Val	
					185					190					195	
	Cys	Leu	Arg	Gln	Leu	Leu	Thr	Phe	Phe	Glu	Lys	Ala	Ala	Glu	Pro	
					200					205					210	
25	His	Ala	Gln	Gly	Leu	Leu	Leu	Cys	Pro	Cys	Ala	Pro	Asn	Asp	Arg	
					215					220					225	
	Gly	Cys	Gly	Glu	Arg	Arg	Arg	Asn	Thr	Ile	Ala	Pro	Asn	Cys	Ala	
					230					235					240	
30	Leu	Pro	Pro	Val	Ala	Pro	Asn	Cys	Leu	Glu	Leu	Arg	Arg	Leu	Cys	
					245					250					255	
	Phe	Ser	Asp	Pro	Leu	Cys	Arg	Ser	Arg	Leu	Val	Asp	Phe	Gln	Thr	
					260					265					270	
	His	Cys	His	Pro	Met	Asp	Ile	Leu	Gly	Thr	Cys	Ala	Thr	Glu	Gln	
					275					280					285	
35	Ser	Arg	Cys	Leu	Arg	Ala	Tyr	Leu	Gly	Leu	Ile	Gly	Thr	Ala	Met	
					290					295					300	
	Thr	Pro	Asn	Phe	Val	Ser	Asn	Val	Asn	Thr	Ser	Val	Ala	Leu	Ser	
					305					310					315	



	Cys Thr Cys Arg Gly Ser Gly Asn Leu Gln Glu Glu Cys Glu Met	320	325	330
	Leu Glu Gly Phe Phe Ser His Asn Pro Cys Leu Thr Glu Ala Ile	335	340	345
5	Ala Ala Lys Met Arg Phe His Ser Gln Leu Phe Ser Gln Asp Trp	350	355	360
	Pro His Pro Thr Phe Ala Val Met Ala His Gln Asn Glu Asn Pro	365	370	375
10	Ala Val Arg Pro Gln Pro Trp Val Pro Ser Leu Phe Ser Cys Thr	380	385	390
	Leu Pro Leu Ile Leu Leu Leu Ser Leu Trp Pro Asp Lys Thr His	395	400	405
	Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser	410	415	420
15	Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser	425	430	435
	Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu	440	445	450
20	Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val	455	460	465
	His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr	470	475	480
	Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu	485	490	495
25	Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro	500	505	510
	Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg	515	520	525
30	Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr	530	535	540
	Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro	545	550	555
	Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn	560	565	570
35	Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe	575	580	585
	Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln	590	595	600

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 35 40 45  
 Asp Leu Pro Val Leu Asp Gln Leu Leu Glu Pro Ser Ser Leu Gln  
 50 55 60  
 Gly Ser Glu Leu His Gly Trp Arg Pro Gln Val Asp Cys Val Arg  
 65 70 75  
 20 Ala Asn Glu Leu Cys Ala Ala Glu Ser Asn Cys Ser Ser Arg Tyr  
 80 85 90  
 Arg Thr Leu Arg Gln Cys Leu Ala Gly Arg Asp Arg Asn Thr Met  
 95 100 105  
 25 Leu Ala Asn Lys Glu Cys Gln Ala Ala Leu Glu Val Leu Gln Glu  
 110 115 120  
 Ser Pro Leu Tyr Asp Cys Arg Cys Lys Arg Gly Met Lys Lys Glu  
 125 130 135  
 Leu Gln Cys Leu Gln Ile Tyr Trp Ser Ile His Leu Gly Leu Thr  
 140 145 150  
 30 Glu Gly Glu Glu Phe Tyr Glu Ala Ser Pro Tyr Glu Pro Val Thr  
 155 160 165  
 Ser Arg Leu Ser Asp Ile Phe Arg Leu Ala Ser Ile Phe Ser Gly  
 170 175 180  
 35 Thr Gly Thr Asp Pro Ala Val Ser Thr Lys Ser Asn His Cys Leu  
 185 190 195  
 Asp Ala Ala Lys Ala Cys Asn Leu Asn Asp Asn Cys Lys Lys Leu  
 200 205 210  
 Arg Ser Ser Tyr Ile Ser Ile Cys Asn Arg Glu Ile Ser Pro Thr  
 215 220 225

	Glu Arg Cys Asn Arg Arg Lys Cys His Lys Ala Leu Arg Gln Phe	230	235	240
	Phe Asp Arg Val Pro Ser Glu Tyr Thr Tyr Arg Met Leu Phe Cys	245	250	255
5	Ser Cys Gln Asp Gln Ala Cys Ala Glu Arg Arg Arg Gln Thr Ile	260	265	270
	Leu Pro Ser Cys Ser Tyr Glu Asp Lys Glu Lys Pro Asn Cys Leu	275	280	285
10	Asp Leu Arg Ser Leu Cys Arg Thr Asp His Leu Cys Arg Ser Arg	290	295	300
	Leu Ala Asp Phe His Ala Asn Cys Arg Ala Ser Tyr Arg Thr Ile	305	310	315
	Thr Ser Cys Pro Ala Asp Asn Tyr Gln Ala Cys Leu Gly Ser Tyr	320	325	330
15	Ala Gly Met Ile Gly Phe Asp Met Thr Pro Asn Tyr Val Asp Ser	335	340	345
	Asn Pro Thr Gly Ile Val Val Ser Pro Trp Cys Asn Cys Arg Gly	350	355	360
20	Ser Gly Asn Met Glu Glu Glu Cys Glu Lys Phe Leu Arg Asp Phe	365	370	375
	Thr Glu Asn Pro Cys Leu Arg Asn Ala Ile Gln Ala Phe Gly Asn	380	385	390
	Gly Thr Asp Val Asn Met Ser Pro Lys Gly Pro Ser Leu Pro Ala	395	400	405
25	Thr Gln Ala Pro Arg Val Glu Lys Thr Pro Ser Leu Pro Asp Asp	410	415	420
	Leu Ser Asp Ser Thr Ser Leu Gly Thr Ser Val Ile Thr Thr Cys	425	430	435
30	Thr Ser Ile Gln Glu Gln Gly Leu Lys Ala Asn Asn Ser Lys Glu	440	445	450
	Leu Ser Met Cys Phe Thr Glu Leu Thr Thr Asn Ile Ile Pro Gly	455	460	465
	Trp Arg Ala Trp Val Pro Val Val Leu Gly Val Leu Thr Ala Leu	470	475	480
35	Val Thr Ala Ala Ala Leu Ala Leu Ile Leu Leu Arg Lys Arg Arg	485	490	495
	Lys Glu Thr Arg Phe Gly Gln Ala Phe Asp Ser Val Met Ala Arg	500	505	510

	Gly Glu Pro Ala Val His Phe Arg Ala Ala Arg Ser Phe Asn Arg	515	520	525
	Glu Arg Pro Glu Arg Ile Glu Ala Thr Leu Asp Ser Leu Gly Ile	530	535	540
5	Ser Asp Glu Leu Lys Glu Lys Leu Glu Asp Val Leu Ile Pro Glu	545	550	555
	Gln Gln Phe Thr Leu Gly Arg Met Leu Gly Lys Gly Glu Phe Gly	560	565	570
10	Ser Val Arg Glu Ala Gln Leu Lys Gln Glu Asp Gly Ser Phe Val	575	580	585
	Lys Val Ala Val Lys Met Leu Lys Ala Asp Ile Ile Ala Ser Ser	590	595	600
	Asp Ile Glu Glu Phe Leu Arg Glu Ala Ala Cys Met Lys Glu Phe	605	610	615
15	Asp His Pro His Val Ala Lys Leu Val Gly Val Ser Leu Arg Ser	620	625	630
	Arg Ala Lys Gly Arg Leu Pro Ile Pro Met Val Ile Leu Pro Phe	635	640	645
20	Met Lys His Gly Asp Leu His Ala Phe Leu Leu Ala Ser Arg Ile	650	655	660
	Gly Glu Asn Pro Phe Asn Leu Pro Leu Gln Thr Leu Ile Arg Phe	665	670	675
	Met Val Asp Ile Ala Cys Gly Met Glu Tyr Leu Ser Ser Arg Asn	680	685	690
25	Phe Ile His Arg Asp Leu Ala Ala Arg Asn Cys Met Leu Ala Glu	695	700	705
	Asp Met Thr Val Cys Val Ala Asp Phe Gly Leu Ser Arg Lys Ile	710	715	720
30	Tyr Ser Gly Asp Tyr Tyr Arg Gln Gly Cys Ala Ser Lys Leu Pro	725	730	735
	Val Lys Trp Leu Ala Leu Glu Ser Leu Ala Asp Asn Leu Tyr Thr	740	745	750
	Val Gln Ser Asp Val Trp Ala Phe Gly Val Thr Met Trp Glu Ile	755	760	765
35	Met Thr Arg Gly Gln Thr Pro Tyr Ala Gly Ile Glu Asn Ala Glu	770	775	780
	Ile Tyr Asn Tyr Leu Ile Gly Gly Asn Arg Leu Lys Gln Pro Pro	785	790	795

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      Glu Cys Met Glu Asp Val Tyr Asp Leu Met Tyr Gln Cys Trp Ser
              800                      805                      810

      Ala Asp Pro Lys Gln Arg Pro Ser Phe Thr Cys Leu Arg Met Glu
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5     Leu Glu Asn Ile Leu Gly Gln Leu Ser Val Leu Ser Ala Ser Gln
              830                      835                      840

      Asp Pro Leu Tyr Ile Asn Ile Glu Arg Ala Glu Glu Pro Thr Ala
              845                      850                      855

10    Gly Gly Ser Leu Glu Leu Pro Gly Arg Asp Gln Pro Tyr Ser Gly
              860                      865                      870

      Ala Gly Asp Gly Ser Gly Met Gly Ala Val Gly Gly Thr Pro Ser
              875                      880                      885

      Asp Cys Arg Tyr Ile Leu Thr Pro Gly Gly Leu Ala Glu Gln Pro
              890                      895                      900

15    Gly Gln Ala Glu His Gln Pro Glu Ser Pro Leu Asn Glu Thr Gln
              905                      910                      915

      Arg Leu Leu Leu Leu Gln Gln Gly Leu Leu Pro His Ser Ser Cys
              920                      925                      930

20    Ala Asp Ala Ser Leu Lys Met Ala Asp Pro Asn Arg Phe Arg Gly
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      Lys Asp Leu Pro Val Leu
              950 951

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      Asp Ala Ser Leu Lys Met Ala Asp Pro Asn Arg Phe Arg Gly Lys
          35           40           45

35    Asp Leu Pro Val Leu Asp Gln Leu Leu Glu Ala Gly Asn Ser Leu
          50           55           60

      Ala Thr Glu Asn Arg Phe Val Asn Ser Cys Thr Gln Ala Arg Lys
          65           70           75

      Lys Cys Glu Ala Asn Pro Ala Cys Lys Ala Ala Tyr Gln His Leu
          80           85           90

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	Gly Ser Cys Thr Ser Ser Leu Ser Arg Pro Leu Pro Leu Glu Glu	95	100	105
	Ser Ala Met Ser Ala Asp Cys Leu Glu Ala Ala Glu Gln Leu Arg	110	115	120
5	Asn Ser Ser Leu Ile Asp Cys Arg Cys His Arg Arg Met Lys His	125	130	135
	Gln Ala Thr Cys Leu Asp Ile Tyr Trp Thr Val His Pro Ala Arg	140	145	150
10	Ser Leu Gly Asp Tyr Glu Leu Asp Val Ser Pro Tyr Glu Asp Thr	155	160	165
	Val Thr Ser Lys Pro Trp Lys Met Asn Leu Ser Lys Leu Asn Met	170	175	180
	Leu Lys Pro Asp Ser Asp Leu Cys Leu Lys Phe Ala Met Leu Cys	185	190	195
15	Thr Leu His Asp Lys Cys Asp Arg Leu Arg Lys Ala Tyr Gly Glu	200	205	210
	Ala Cys Ser Gly Ile Arg Cys Gln Arg His Leu Cys Leu Ala Gln	215	220	225
20	Leu Arg Ser Phe Phe Glu Lys Ala Ala Glu Ser His Ala Gln Gly	230	235	240
	Leu Leu Leu Cys Pro Cys Pro Pro Glu Asp Ala Gly Cys Gly Glu	245	250	255
	Arg Arg Arg Asn Thr Ile Ala Pro Ser Cys Ala Leu Pro Ser Val	260	265	270
25	Thr Pro Asn Cys Leu Asp Leu Arg Ser Phe Cys Arg Ala Asp Pro	275	280	285
	Leu Cys Arg Ser Arg Leu Met Asp Phe Gln Thr His Cys His Pro	290	295	300
30	Met Asp Ile Leu Gly Thr Cys Ala Thr Glu Gln Ser Arg Cys Leu	305	310	315
	Arg Ala Tyr Leu Gly Leu Ile Gly Thr Ala Met Thr Pro Asn Phe	320	325	330
	Ile Ser Lys Val Asn Thr Thr Val Ala Leu Ser Cys Thr Cys Arg	335	340	345
35	Gly Ser Gly Asn Leu Gln Asp Glu Cys Glu Gln Leu Glu Arg Ser	350	355	360
	Phe Ser Gln Asn Pro Cys Leu Val Glu Ala Ile Ala Ala Lys Met	365	370	375

	Arg Phe His Arg Gln Leu Phe Ser Gln Asp Trp Ala Asp Ser Thr	380	385	390
	Phe Ser Val Val Gln Gln Gln Asn Ser Asn Pro Ala Trp Arg Ala	395	400	405
5	Trp Val Pro Val Val Leu Gly Val Leu Thr Ala Leu Val Thr Ala	410	415	420
	Ala Ala Leu Ala Leu Ile Leu Leu Arg Lys Arg Arg Lys Glu Thr	425	430	435
10	Arg Phe Gly Gln Ala Phe Asp Ser Val Met Ala Arg Gly Glu Pro	440	445	450
	Ala Val His Phe Arg Ala Ala Arg Ser Phe Asn Arg Glu Arg Pro	455	460	465
	Glu Arg Ile Glu Ala Thr Leu Asp Ser Leu Gly Ile Ser Asp Glu	470	475	480
15	Leu Lys Glu Lys Leu Glu Asp Val Leu Ile Pro Glu Gln Gln Phe	485	490	495
	Thr Leu Gly Arg Met Leu Gly Lys Gly Glu Phe Gly Ser Val Arg	500	505	510
20	Glu Ala Gln Leu Lys Gln Glu Asp Gly Ser Phe Val Lys Val Ala	515	520	525
	Val Lys Met Leu Lys Ala Asp Ile Ile Ala Ser Ser Asp Ile Glu	530	535	540
	Glu Phe Leu Arg Glu Ala Ala Cys Met Lys Glu Phe Asp His Pro	545	550	555
25	His Val Ala Lys Leu Val Gly Val Ser Leu Arg Ser Arg Ala Lys	560	565	570
	Gly Arg Leu Pro Ile Pro Met Val Ile Leu Pro Phe Met Lys His	575	580	585
30	Gly Asp Leu His Ala Phe Leu Leu Ala Ser Arg Ile Gly Glu Asn	590	595	600
	Pro Phe Asn Leu Pro Leu Gln Thr Leu Ile Arg Phe Met Val Asp	605	610	615
	Ile Ala Cys Gly Met Glu Tyr Leu Ser Ser Arg Asn Phe Ile His	620	625	630
35	Arg Asp Leu Ala Ala Arg Asn Cys Met Leu Ala Glu Asp Met Thr	635	640	645
	Val Cys Val Ala Asp Phe Gly Leu Ser Arg Lys Ile Tyr Ser Gly	650	655	660

	Asp	Tyr	Tyr	Arg	Gln	Gly	Cys	Ala	Ser	Lys	Leu	Pro	Val	Lys	Trp	665	670	675
	Leu	Ala	Leu	Glu	Ser	Leu	Ala	Asp	Asn	Leu	Tyr	Thr	Val	Gln	Ser	680	685	690
5	Asp	Val	Trp	Ala	Phe	Gly	Val	Thr	Met	Trp	Glu	Ile	Met	Thr	Arg	695	700	705
	Gly	Gln	Thr	Pro	Tyr	Ala	Gly	Ile	Glu	Asn	Ala	Glu	Ile	Tyr	Asn	710	715	720
10	Tyr	Leu	Ile	Gly	Gly	Asn	Arg	Leu	Lys	Gln	Pro	Pro	Glu	Cys	Met	725	730	735
	Glu	Asp	Val	Tyr	Asp	Leu	Met	Tyr	Gln	Cys	Trp	Ser	Ala	Asp	Pro	740	745	750
	Lys	Gln	Arg	Pro	Ser	Phe	Thr	Cys	Leu	Arg	Met	Glu	Leu	Glu	Asn	755	760	765
15	Ile	Leu	Gly	Gln	Leu	Ser	Val	Leu	Ser	Ala	Ser	Gln	Asp	Pro	Leu	770	775	780
	Tyr	Ile	Asn	Ile	Glu	Arg	Ala	Glu	Glu	Pro	Thr	Ala	Gly	Gly	Ser	785	790	795
20	Leu	Glu	Leu	Pro	Gly	Arg	Asp	Gln	Pro	Tyr	Ser	Gly	Ala	Gly	Asp	800	805	810
	Gly	Ser	Gly	Met	Gly	Ala	Val	Gly	Gly	Thr	Pro	Ser	Asp	Cys	Arg	815	820	825
	Tyr	Ile	Leu	Thr	Pro	Gly	Gly	Leu	Ala	Glu	Gln	Pro	Gly	Gln	Ala	830	835	840
25	Glu	His	Gln	Pro	Glu	Ser	Pro	Leu	Asn	Glu	Thr	Gln	Arg	Leu	Leu	845	850	855
	Leu	Leu	Gln	Gln	Gly	Leu	Leu	Pro	His	Ser	Ser	Cys	Ala	Asp	Ala	860	865	870
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	Pro	Val	Leu													888		

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>C12N 15/12, C07K 14/71, C12N 15/85, 15/70, 15/81, 1/19, 5/10, 1/21, C07K 19/00, 16/28, G01N 33/68, C12Q 1/42, A61K 39/395</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 99/49039</b> <b>(43) International Publication Date:</b> 30 September 1999 (30.09.99)
<b>(21) International Application Number:</b> PCT/US99/06098 <b>(22) International Filing Date:</b> 19 March 1999 (19.03.99) <b>(30) Priority Data:</b> 60/079,124                      23 March 1998 (23.03.98)                      US 60/081,569                      13 April 1998 (13.04.98)                      US <b>(71) Applicant:</b> GENENTECH, INC. [US/US]; 1 DNA Way, South San Francisco, CA 94080-4990 (US). <b>(72) Inventors:</b> DE SAUVAGE, Frederic, J.; 187 Shooting Star Isle, Foster City, CA 94404 (US). KLEIN, Robert, D.; 1044 Webster Street, Palo Alto, CA 94301 (US). PHILLIPS, Heidi, S.; 15 Pine Avenue, San Carlos, CA 94070 (US). ROSENTHAL, Arnon; 40 Tulip Court, Burlingame, CA 94010 (US). <b>(74) Agents:</b> TORCHIA, Timothy, E. et al.; Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080-4990 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>  <b>(88) Date of publication of the international search report:</b> 10 February 2000 (10.02.00)
<b>(54) Title:</b> GFR $\alpha$ 3 AND ITS USES  <b>(57) Abstract</b> <p>The present invention relates to nucleotide sequences, including expressed sequence tags (ESTS'S), oligonucleotide probes, polypeptides, vectors and host cells expressing, and immunoadhesions and antibodies to mammalian GFR<math>\alpha</math>3, a x-subunit receptor of the GDNF (i.e. GFR) receptor family. It further relates to an assay for measuring activation of an x-subunit receptor by detecting tyrosine kinase receptor activation (i.e., autophosphorylation) or other activities related to ligand-induced x-subunit receptor homo-dimerization or homo-oligomerization.</p>		

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<b>EE</b>	Estonia						

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06098

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C07K14/71 C12N15/85 C12N15/70 C12N15/81  
 C12N1/19 C12N5/10 C12N1/21 C07K19/00 C07K16/28  
 G01N33/68 C12Q1/42 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 97 44356 A (BIOGEN, INC.)            27 November 1997 (1997-11-27)            page 2, line 10 - line 19            page 2, line 24 -page 5, line 2; figures            9,10            page 11, line 15 - line 29            page 16, line 5 - line 16            page 31, line 15 -page 34, line 17            see SEQ ID NO:20 and 21</p> <p style="text-align: center;">--- -/--</p>	1-25



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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- \*P\* document published prior to the international filing date but later than the priority date claimed

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- \* & \* document member of the same patent family

Date of the actual completion of the international search

30 November 1999

Date of mailing of the international search report

17. 12. 99

Name and mailing address of the ISA

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Authorized officer

Montero Lopez, B

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06098

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>SHUQIAN JING ET AL.: "GFRalpha-2 and GFRalpha-3 are two new receptors for ligands of the GDNF family" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 52, 26 December 1997 (1997-12-26), pages 33111-33117, XP002065824 MD US abstract page 33111, right-hand column, last paragraph -page 33112, left-hand column, paragraph 3 page 33112, right-hand column, last paragraph -page 33114, left-hand column, paragraph 2</p> <p>---</p>	1-25
X	<p>WINFIRE KAMMER ET AL.: "Homodimerization of Interleukin-4 receptor alpha chain can induce intracellular signalling" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 39, 27 September 1996 (1996-09-27), pages 23634-23637, XP002124368 MD US abstract page 23635, right-hand column, last paragraph -page 23636, left-hand column, paragraph 1</p> <p>---</p>	26
X	<p>LENA CLAEISSON-WELSH: "Signal transduction by the PDGF receptors" PROGRESS IN GROWTH FACTOR RESEARCH, vol. 5, no. 1, 1994, pages 37-54, XP000856759 abstract page 39, last paragraph -page 40, paragraph 2</p> <p>---</p>	26,28,29
A	<p>WO 97 33912 A (GENENTECH INC ;KLEIN ROBERT D (US); MOORE MARK W (US); ROSENTHAL A) 18 September 1997 (1997-09-18) page 3, line 17 -page 5, line 3 page 14, line 12 -page 26, line 12 page 29, line 1 -page 34, line 7 page 43, line 18 -page 44, line 18 page 51, line 14 -page 57, line 8; examples</p> <p>---</p> <p style="text-align: center;">-/--</p>	1-66

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 99/06098

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	NORIIHIKO NAKAMURA ET AL.: "An epidermal growth factor receptor/Jak2 Tyrosine kinase domain chimera induces tyrosine phosphorylation of Stat5 and transduces a growth signal in hematopoietic cells" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 32, 9 August 1996 (1996-08-09), pages 19483-19488, XP002054015 MD US abstract page 19484, right-hand column, paragraph 5 -page 19485, left-hand column, paragraph 1 page 19485, left-hand column, last paragraph -right-hand column, paragraph 1 page 19487, left-hand column, paragraph 2 page 19488, left-hand column, paragraph 2 ----	26-66
P,X	WO 98 53069 A (HUMAN GENOME SCIENCES INC ;NI JIAN (US); GENTZ REINER L (US); HSU) 26 November 1998 (1998-11-26) page 4, line 3 - line 13 page 6, line 28 -page 7, line 4; figure 7 page 8, line 3 - line 13 page 10, line 9 -page 12, line 9 page 18, line 11 - line 24 page 28, line 10 - line 19 page 29, line 23 -page 30, line 19 page 31, line 6 -page 32, line 10 page 35, line 4 - line 14; examples 7,9,10 ----	1-25
P,X	WO 98 54213 A (AMGEN INC.) 3 December 1998 (1998-12-03) page 6, line 13 -page 7, line 6 page 7, line 26 - line 35 page 8, line 9 - line 20 page 11, line 30 - line 31; figure 15 page 15, line 36 -page 17, line 4 page 23, line 37 -page 32, line 30 page 42, line 9 - line 22; example 11 see sequence listing SEQ ID NOs: 37 and 38 ----	1-19, 23-25
P,X	EP 0 846 764 A (SMITHKLINE BEECHAM PLC) 10 June 1998 (1998-06-10)  page 2, line 44 -page 3, line 7 page 3, line 36 -page 5, line 51 page 6, line 50 -page 7, line 11 -----	1,2, 4-17,19, 23-25

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US 99/06098

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
  
Although claim 25 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-25

Nucleic acid encoding a GFRalpha3 polypeptide, analogs thereof; vector and host cell comprising the same and use thereof for producing GFRalpha3 polypeptides; a GFRalpha3 polypeptide, analogs and chimeric molecules comprising the same; antibody binding a GFRalpha3 polypeptide and its use to treat a neuronal disorder of the periphery.

2. Claims: 26-66

Polypeptide comprising a ligand-binding domain of an alpha-subunit receptor and uses thereof for measuring phosphorylation and agonist binding.



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 99/06098

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9744356	A	27-11-1997	AU 3472997	A	09-12-1997
			BG 102973	A	31-08-1999
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WO 9854213	A	03-12-1998	AU 7165998	A	30-12-1998
EP 0846764	A	10-06-1998	CA 2216625	A	27-05-1998
			JP 10337189	A	22-12-1998